Study of the range of antibody levels and activities of *Acinetobacter calcoaceticus – Acinetobacter baumannii* complex and *Haemophilus influenzae* Lipopolysaccharides.

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Abstract

Hospital acquired pneumonia is a major problem in the nosocomial environment worldwide. The rise in the number and level of antibiotic resistant strains of bacteria means that conventional therapies are no longer as effective as they once were. Many of the main causative organisms are Gram-negative rods, such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Haemophilus influenzae* and one that has become a greater problem in the last twenty years *Acinetobacter genospecies 13 TU*. Lipopolysaccharide (LPS) is a molecule that is found on the cell surface of all Gram-negative organisms. LPS is a vital part of the outer membrane of Gram-negative bacteria and is a major factor in these organisms' ability to cause serious infection and disease. While many Gram-negative organisms, such as *E. coli* and *Klebsiella pneumoniae,* are well characterised, other species that have become potential nosocomial pathogens more recently, such as *Acinetobacter genospecies 13 TU,* are much less well characterised. It is unknown as to how widespread exposure to *Acinetobacter genospecies 13 TU* is in a healthy population. Also, little is also about pathogenesis of *Acinetobacter genospecies 13 TU* such as the capacity for induction of cytokines by the *Acinetobacter genospecies 13 TU* LPS

LPS was extracted with the aqueous phenol method and re-purified by Voegel's method from eight strains of *Acinetobacter genospecies 13 TU,* four strains of *Haemophilus influenzae*, two strains of *Pseudomonas aeruginosa*, two strains of *Klebsiella pneumoniae* and two strains of *E. coli*. These LPSs were used in enzyme linked immunosorbant assays (ELISAs) with serum taken from 475 blood donors from the Southeast Scotland Blood Transfusion Service. The results from the ELISAs

were averaged for each individual blood donor across all the species tested. These averaged results were compared across the species.

LPS from two strains of each species, ten in all, were used to challenge the THP-1 human monocytic cell line and the mRNA was extracted and used in quantitative polymerase chain reactions to measure cytokine induction.

It was seen that exposure to *Acinetobacter genospecies 13 TU* LPS is about as widespread in a healthy population from Southeast Scotland as exposure to *Pseudomonas aeruginosa* LPS and somewhat similar to *Klebsiella pneumoniae* LPS. Antibodies to *E. coli* LPS and *Haemophilus influenzae* LPS were similarly widespread in a healthy population from Southeast Scotland. These last two were much more widely spread than the other organisms tested. Some individuals seem to produce antibodies at high levels to all of the LPSs tested. It may be possible to use serum from these individuals to make a hyper-immune immunoglobulin preparation to be used in the immunotherapy of hospital associated pneumonia. The LPS from one of the strains of *Acinetobacter genospecies 13 TU* was able to induce similar levels of cytokine production as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. It was able to induce higher levels of cytokine production over a greater number of cytokines than both *Haemophilus influenzae* and *E. coli* LPS. LPS from the other strain of *Acinetobacter genospecies 13 TU* tested induced lower levels of cytokines compared to the other strain. These levels were lower than those developed by *Haemophilus influenzae* and *E. coli* LPS as well as those induced by the LPSs from *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. It seems that there is a range of different levels of cytokine production induced by *Acinetobacter genospecies 13 TU* LPS with some strains inducing high levels and others inducing low levels of cytokines.

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Abbreviations

A. g 13 TU - *Acinetobacter* genospecies 13 TU

AME - Aminoglycoside modifying enzyme

BIP – bactericidal/permeability increasing protein

BHI - Brain heart infusion

CAP - Community acquired pneumonia

CD- cluster of differentitation

cDNA - complimentary DNA

Ct - cycle threshold

DNA - deoxyribonucleic acid

DnTP - deoxynucleoside

E.c. - *Escherichia coli*

E. coli - *Escherichia coli*

ECD - Ecto domains

ELISA - Enzyme linked immunosorbant assay

ESBL - Extended spectrum β-lactamase

FBS - Foetal Bovine serum

GLcN - Glucosamine

Glu - Glutamine

HAP - Hospital acquired pneumonia

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H.i. - *Haemophilus influenzae*

ICU - Intensive care unit

Ig - Immunoglobulin

IL - Interleukin

IFN - Interferon

IRAK - IL-1 receptor-associated kinases

Kdo - 3-deoxy-D-*manno*-oct-2-ulosonic acid

Ko - 2-keto-D-glycerol-D-talo-octonic acid

K.p. - *Klebsiella pneumoniae*

LAL - Limulus amoebocyte lysate

LBP - Lipopolysaccharide binding protein

LPS - Lipopolysaccharide

LRR - Leucine rich repeats

MAP - Mitogen-activated protein

mCD-14 – membrane CD-14

MD-2 - Myeloid differentiation protein

MRSA - methicillin resistant *Staphylococcus aureus*

NF-κB - Nuclear factor- κB

OD - Optical density

OMP - Outer membrane protein

P.a. - *Pseudomonas aeruginosa*

PAGE - Polyacrylamide gel electrophoresis

PAMP - Pathogen associated molecular pattern

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PF - Pyrogen free

PRR - Pattern recognition receptors

PS+ G - Penicillin and Streptomycin + L-Glutamine

QPCR - Quantitative polymerase chain reaction

RIE - Royal Infirmary Edinburgh

RNA - ribonucleic acid

RPMI - Roswell Park Memorial Institute

RT - Reverse transcription

RT PCR - Real time polymerase chain reaction

S-form - Smooth form

sCD-14 – soluble CD-14

TIR - Toll/IL-1 receptor homologue

TLR - Toll like receptor

TNF - Tumour necrosis factor

TRAF 6 - TNF receptor associated factor 6

VAP - Ventilator associated pneumonia

Declaration

All experimental procedures in this thesis were carried out by the author unless stated otherwise

1 Introduction

1.1 Lipopolysaccharide (LPS)

1.1.1 Overview

One of the main differences between Gram-positive and Gram-negative bacterial cell walls is that Gram-negative organisms have an outer and an inner membrane, whilst Gram-positive organisms have a large peptidoglycan cell wall. Lipopolysaccharide (LPS) is one of the essential components of the outer membrane of Gram-negative bacteria, as is shown in Figure 1.1 below. This LPS is composed of a carbohydrate and a lipid, hence the name lipopolysaccharide (Erridge *et al*, 2002).

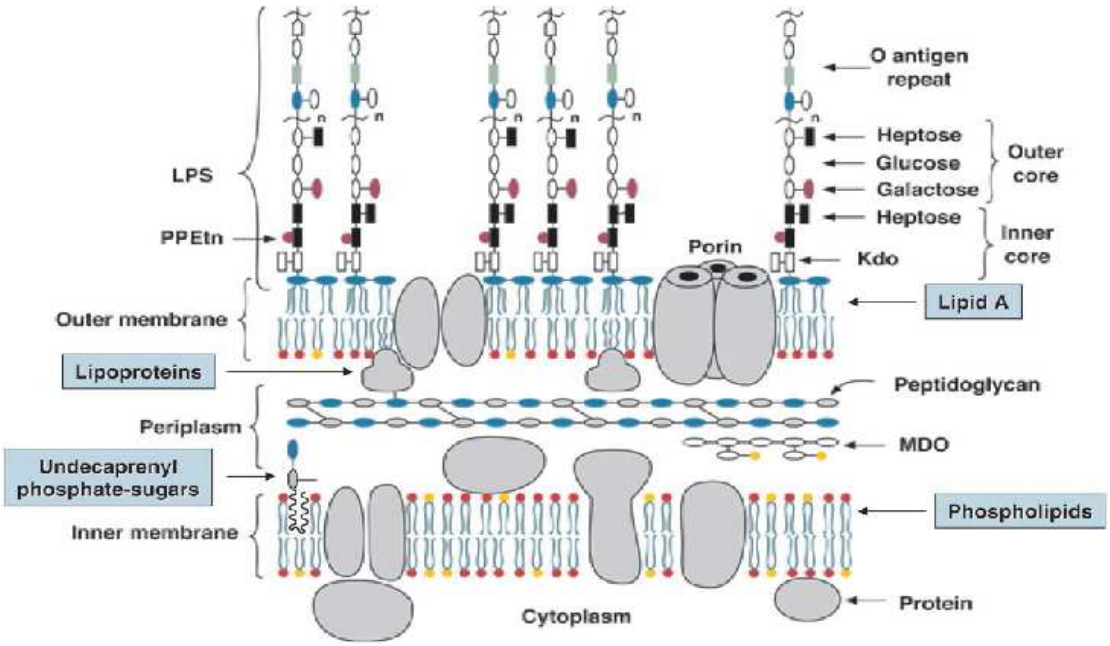


Figure 1.1: The outer membrane of a Gram-negative bacterium. The Lipid A moiety is bound to the outer membrane of the bacterial cell, whilst the O-antigen extrudes into the extra cellular space. (Raetz & Whitfield, 2002)

In 1892 Richard Pfeiffer discovered endotoxin, as a bacterial structure and used it as a basis for paving the way to understanding how micro-organisms cause disease

(Beutler & Rietschel, 2003). Currently it is thought that LPS is essential for the viability of all Gram negative outer membranes, as well as being an extremely potent antagonist of the innate immune system (Alexander & Rietschel, 2001; Dobrovolskaia & Vogel 2002). Thus far, two exceptions have been identified; some bacteria contain glycosphinolipids instead of LPS e.g. *Sphingomonas* (Kawahara *et al*, 1991) and there is an LPS-deficient mutant of *Neisseria meningitidis,* both these organisms have the ability to survive so both are viable (Gronow & Brade, 2001).

The evolution of macrophages and monocytes alongside LPS has meant that the mammalian immune system is equipped with a rapid recognition of and reaction to infection by Gram-negative bacteria. The immune response is traditionally characterised by the production of a wide range of inflammatory mediators, such as TNF-α, INF-γ, Il-1β, IL-8 and Il-10. These, in moderate levels in a local site, are extremely beneficial to the host as they up-regulate inflammation and ready the immune system to fight the infection causing microbes (Erridge *et al*, 2002). However, in conditions where the host suffers an overexposure to LPS or the LPS is found systemically, it can be extremely dangerous as the immune system is over activated and starts to attack the host system. This can lead to severe sepsis and even to multiple organ failure (Prucha *et al*, 2003). LPS is found either as a causative effect or a modulator in a large number of other diseases. Many diseases caused by Gram-negative bacteria are a consequence of the structures of the LPSs.

1.1.2 General Structure of LPS

It is known that each Gram-negative bacterial cell has approximately $3.5x10^6$ molecules of LPS in its outer membrane (Rietschel *et al,* 1994). Each molecule of LPS has a molecular weight of 2000-20000 (Caroff *et al*, 2002). About 75% of the outer bacterial cell surface is made up of LPS, which accounts for 10-15% of the total molecules of the outer membrane. Between 1-3% of the total mass of the cell is LPS.

The structure of LPS is heat-stable and made up of carbohydrate and lipid (Dixon & Darveau, 2005). The linking of a carbohydrate to a lipid is a common method to attach a hydrophilic biopolymer firmly to the outer surface of a membrane (Wilkinson, 1996). LPS represents a family grouping. They all share these characteristics; they are a vital part of the outer membrane of Gram-negative bacteria; they have a phospholid anchor, the lipid A moiety; they are constructed in a similar fashion to each other and all have endotoxic properties. There is also O antigen recognition (Wilkinson, 1996).

All LPS conforms to the same general structural architecture, (Heumann and Roger, 2002). The majority of LPS is composed of three parts; the highly conserved Lipid A moiety, which is embedded into the membrane; the core oligosaccharide and the O-specific polysaccharide chain (Wilkinson, 1996; Erridge *et al*, 2002; Dixon & Darveau, 2005). Whilst the general structure of the LPS is the same, there is a large amount of diversity in the exact composition of the different parts, between species and also between strains of these species. This is mainly due to a high variety of different O-polysaccharide compositions. The core also varies between strains, but far more between species. The Lipid A moiety varies very little, if at all, between strains, but more so between species (Alexander & Rietschel, 2001; Erridge *et al*, 2002;

Dobrovolskaia & Vogel 2002). There are certain conserved domains within LPS, which are vital to the cells' survival as they maintain the integrity or preserve the whole structure of the outer membrane. The variable domains are not vital to the survival of the organism and their lack could be advantageous for the bacteria.

Mostly the changes in the length of the segment will result in small changes to the LPS structure, whereas a major change, such as something affecting the overall chemical construction or the attachment of charged groups can lead to the damaging of the overall structure (Dixon & Davreau, 2005). Typical LPS containing all three segments is found commonly in *Escherichia coli* and many other bacterial species. It is often called smooth or S-form chemotype (Poxton, 1995; Wilkinson, 1996 and Beutler & Rietschel, 2003). So far only one group of Gram negative wild type bacteria has been identified, which does not express LPS; these belong to the genus *Sphingomonas* and they express a glycosphinolipid on their outer membrane in place of LPS (Kawahara *et al*, 1991)

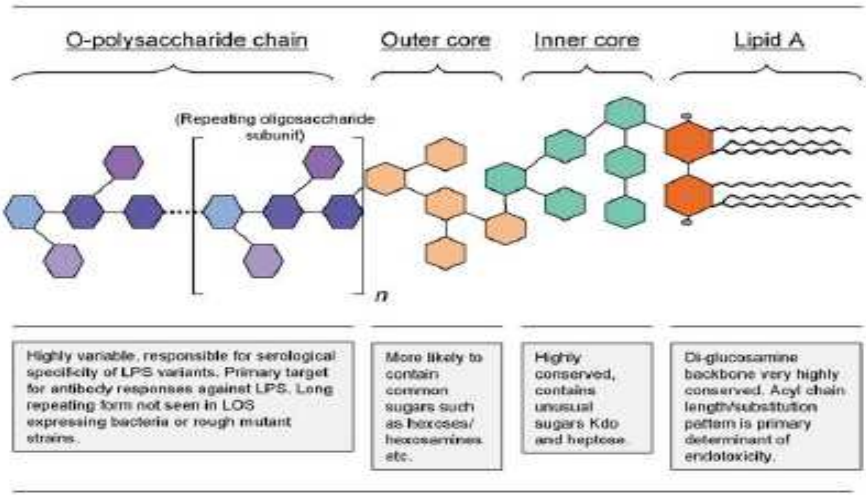


Figure 1.2: General structure of Gram negative LPS (Erridge *et al*, 2002)

1.1.2.1 O-Polysaccharide chain

LPS which has an O-polysaccharide chain, also called an O-antigen, is referred to as smooth LPS. Smooth LPSs are found on members of the Enterobacteriaceae, Pseudomonadaceae, Pasturellaceae and Vibrionaceae along with many other Gram negative species. There is an incredible range of diversity in the O-antigen, with over 60 different monosaccharides and 30 different non-carbohydrate components being recognised so far (Raetz & Whitfield, 2002). The O-antigen is made up of several repeating units, which can consist of between 1 and 8 of the monosaccharides mentioned above and can contain or not non-carbohydrate components, as well as having different positions and stereochemistry of the constituents (Erridge *et al*, 2002; Raetz & Whitfield 2002; Caroff *et al*, 2002). The way in which the O-antigen repeating units are arranged varies considerably. They can be linear or branched; there are even some with two different O-antigens attached to the Lipid A moiety i.e. *Pseudomonas aeruginosa* (Kintz & Goldberg 2008). There is also homopolymery (where only one type of monosaccharide is used) or the more common heteropolymery (where many different monosaccharides are used). It is also possible for non-stoichiometric modifications, such as glycosylation or O-acetylation, to change the O-antigen (Raetz & Whitfield, 2002).

All this leads to there being almost limitless variability between the O-antigens inside species. There are more than 170 O serotypes within *Escherichia coli* for example (Raetz & Whitfield, 2002). Other species though exhibit a much smaller variation amongst their O-antigens e.g. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex (Wilkinson, 1996). This massive variation between O-

antigens and the fact that there can be highly specific antibody responses raised against them means that it is possible to categorise the strains by their O-antigen (Erridge *et al*, 2002).

The O-polysaccharide chain is the outermost part of the LPS and also of the cell, thus it is the main part that the immune system acts against. The O-antigen's main function is to protect the bacterial cell from host defences, like bile acids and cationic peptides (Alexander & Rietschel, 2001). It has also been linked to protection from the alternative complement cascade. The O-antigen protects the bacterium by interfering with the assembly of the membrane attack complex (Raetz & Whitfield, 2002). The complement appears to bind to the O-antigen and, owing to being further out from the cell membrane is unable to form the pores to destroy the cells. Short O-antigen on the rough LPS types does not offer the same protection that the smooth LPS types offer (Lerouge & Vanderleyden, 2002). Chain length has also been associated with ability of *Escherichia coli* to survive neutrophil bactericidal/permeability increasing protein (BIP) (Weiss *et al*, 1986). It has also been observed that the ability of *Vibrio cholerae* rough LPS mutants to colonise the intestinal epithelium is impaired. This is due to increased sensitivity to cationic peptides and complement (Nesper *et al*, 2001).

It has been observed in some species that the O-antigen is very similar to host antigen. This is protective, as the host's immune system is unable to identify the bacterial cell and thus it is able to survive e.g. this has been observed in *Helicobacter pylori* (Appelmelk *et al*, 2000). A large number of wild type pathogenic gram negative bacterial species have naturally rough LPS, for example *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae* or *Bordetella pertussis* (Erridge *et al*, 2002). These species easily colonise the mucosal surfaces of the respiratory and

urogenital tracts. It seems that these bacteria express different terminal oligosaccharide domains, which are very similar to human glycosphinolipids (Erridge *et al*, 2002). The immunogenicity of the O-antigen is dependent on the adjuvant effect of the Lipid-A component, as O-antigen on its own is not very immunogenic (Reeves, 1995).

1.1.2.2 Core polysaccharide

The oligosaccharide core of LPS is much more conserved compared with the O-antigen. An example of this is that *E. coli* has only five distinct core regions (R1, R2, R3, R4, K12) as is shown in the figure 1.3 below, compared with over 170 O-antigens so far identified (Dobrovolskaia & Vogel, 2002; Erridge *et al*, 2002).

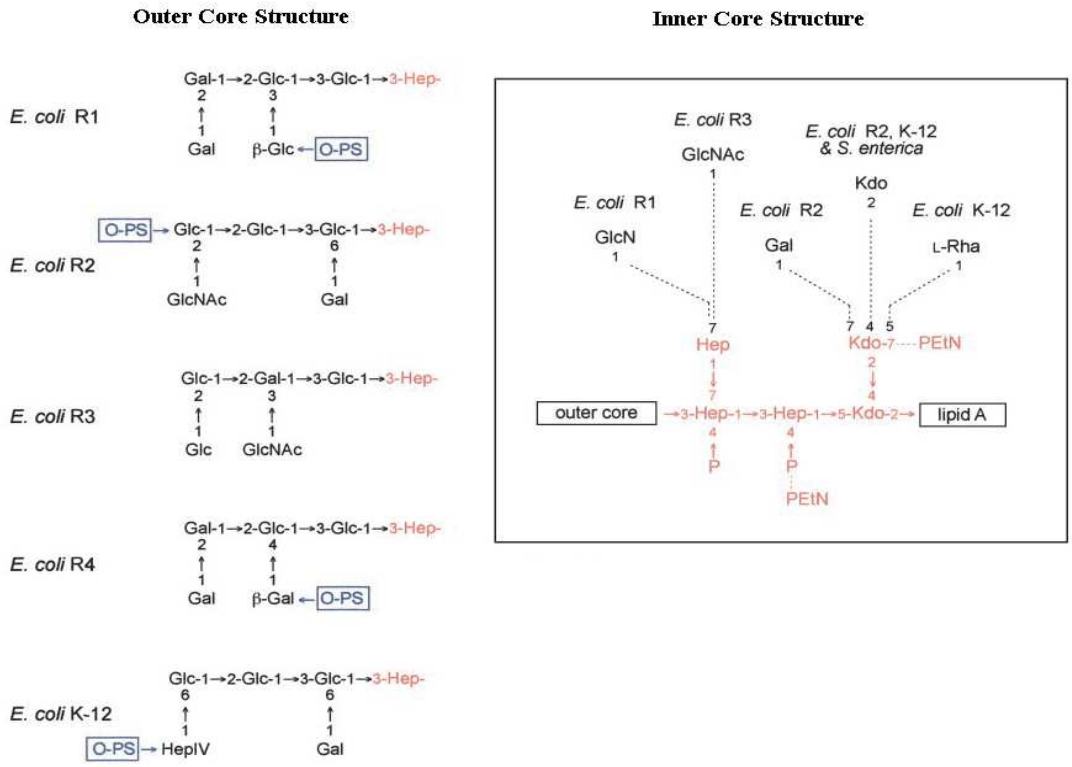


Figure 1.3: The sugar makeup of the five core structures of *E. coli*. The figure shows the difference in the different *E. coli* core types. It can been seen that the inner core varies less than the outer core (Raetz & Whitfield, 2002).

The core is madeup of two components the inner and outer cores. The outer normally consists of common hexose sugars, such as glucose, galactose and N-acetyl galactosamine. This is sometimes referred to as the hexose region. This region is mostly considered more variable in nature compared with the inner core (Wilkinson, 1996; Erridge *et al*, 2002; Raetz & Whitfield 2002).

The inner core consists of much rarer sugars, such as 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) or heptose. Kdo has so far been found in all cores bound to the carbohydrate backbone of the Lipid A moiety. Only two exceptions have been found so far, those of the *Burkholderia cepacia* complex and *Acinetobacter* species, which both have a derivative of Kdo, 2-keto-D-glycerol-D-talo-octonic acid (Ko) instead (Wilkinson, 1996; Vinogradov *et al*, 2002; Erridge *et al*, 2002; Vinion-Dubiel & Goldberg, 2003).

It is widely held that Kdo is vital for the viability of Gram-negative bacteria. This is supported by the fact that the shortest rough mutant, *Haemophilus influenzae* strain I-$^{69-}$/b^{+}, consists only of a kdo residue attached to the Lipid A moiety (Helander *et al*, 1988). Also *Chlamydia* species, which have the shortest naturally occurring core known, consists of only 3 Kdo residues (Brade & Brade, 1987). Both of these indicate that a Kdo residue bound to the Lipid A moiety is vital for cell structure stability (Raetz & Whitfield, 2002; Erridge *et al*, 2002; Dobrovolskaia & Vogel 2002).

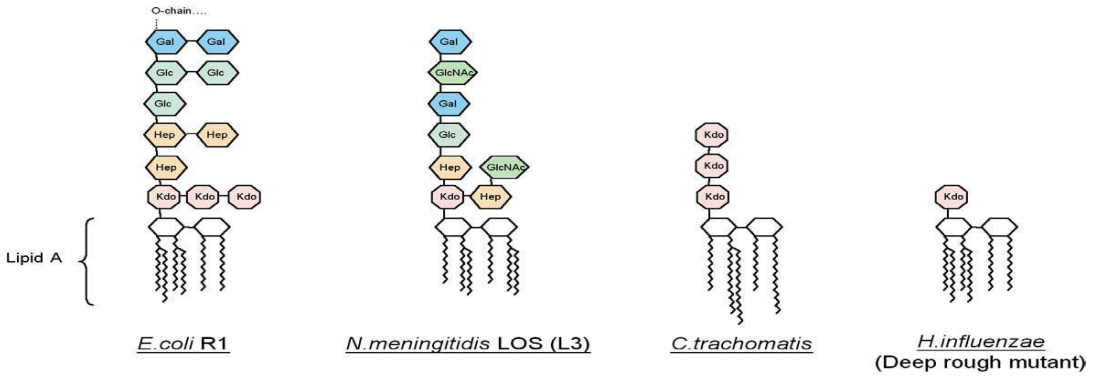


Figure 1.4: The structure of the core region of various Gram-negative bacterial pathogens. This is a set of examples of different types of core polysaccharides. The *E. coli* is the rough mutant R1 and it can be seen to have both an inner and outer region with both heptose and Kdo in the inner core. The *Neisseria meningitidis* is the entire LOS region of the organism. All isolates of *Chlamydia trachomatis* so far investigated have been found with only these three Kdos. The *Haemophilus influenzae* deep rough mutant has the shortest core seen for a viable Gram negative bacteria (Erridge *et al*, 2002).

The sugars of the inner and outer cores can be replaced with charged groups, such as phosphate, pyrophosphate, 2-aminoethylphosphate and others. These charged groups are suspected to have a close association with the Ca^{2+} and Mg^{2+} ions required for membrane structure and function (Rietschel *et al*, 1994).

The inner core's highly conserved nature is ideal for targeting with monoclonal antibodies, which would be able to bind to a wide selection of *E. coli* and *Salmonella* LPS (Di Padova *et al*, 1993). It is also possible to try to identify a common epitope or epitopes and produce a vaccine that will provoke a highly cross-reactive response in human sera (Bennett-Guerrero *et al*, 2000). It is hoped that these would aid in preventing illness in intensive care units.

1.1.2.3 Lipid A Moiety

Lipid A is a vital structural element of LPS, which also is the main site of interaction of LPS with the innate immune system (Cadenas & Cadenas, 2002). It has been hypothesised since the 1950s that the Lipid A moiety is the cause of the endotoxic activity of the LPS. This was demonstrated by Galanos *et al* (1985), when they showed that synthetic lipid A had identical biological activity to *E. coli* lipid A. This proved that the Lipid A moiety is the part of the molecule responsible for endotoxic activity.

The Lipid A moiety is composed of a β-D-GlcN(1-6)-α-D-GlcN disaccharide carrying two phosphoryl groups (at positions 1 and 4'). The two phosphates can be replaced with other groups, such as, ethanolamine, ethanolamine phosphate, ethanolamine diphosphate or 4-amino-4-deoxy-L-arabinopyranose for example. There are up to four acyl chains attached to this structure by ester or amide linkage. The acyl chains can be substituted with further fatty acids, allowing LPS molecules to have up to seven acyl substituents. These can vary greatly between species in nature, number, length, order and saturation (Rosner *et al*, 1979; Galanos *et al*, 1984; Tanamoto *et al*. 1984 Erwin & Munford, 1990; Rietschel *et al*, 1994; Erridge *et al*, 2002; Dobrolskaia & Voegel, 2002; Dixon & Darveau, 2005). The typical structure of an *E. coli* Lipid A can be seen in the figure below.

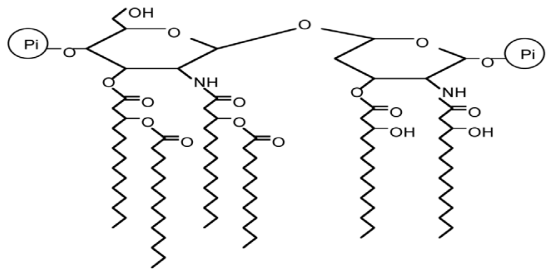


Figure 1.5 *E. coli* Lipid A (Erridge *et al*, 2002).

The chemical structure of *E. coli* Lipid A, is widely thought that this structure is close to that initially identified by human cellular receptors. Deviations from this six acyl chain form are typically less biologically active. (Erridge *at al*, 2002)

Evidence uncovered seems to indicate that the receptor-binding specificity of Lipid A is determined by its hydrophilic region i.e. the phosphorylated D-glucosamine disaccharide. On the other hand the main site of immune cell activation is thought to be mostly controlled by the hydrophobic region i.e. the acyl groups (Rietschel *et al*, 1994; Alexander & Rietschel, 2001)

The highly conserved nature of Lipid A within all species, could lead to the conclusion that there might be a high degree of similarity between the levels of the endotoxic activities. This assumption though is most certainly not the common state of affairs according to the current evidence. It has been observed that differences in Lipid A function are normal and these differences closely affect the endotoxicity of the Lipid A. The dissimilarities are based on structural domains, such as, which hexosamine is found and the extent, type and architecture of the acyl groups attached (Rietschel *et al*, 1994; Netea *et al*, 2002).

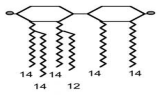
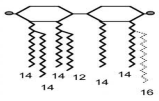
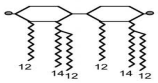
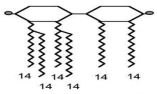
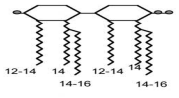
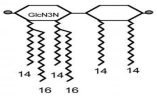
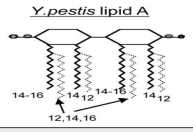
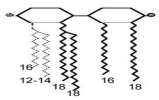
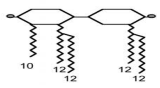
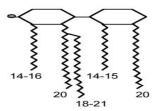
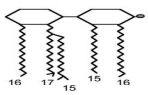
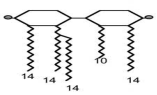
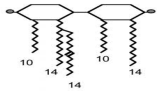
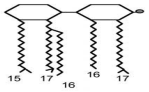
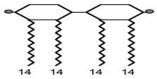
E.coli lipid A	*S.minnesota* lipid A	*N.meningitidis* lipid A	*H.influenzae* lipid A
			
Endotoxic Activity: **+++**	Endotoxic Activity: **+++**	Endotoxic Activity: **+++**	Endotoxic Activity: **+++**
K.pneumoniae lipid A	*C.jejuni* lipid A	*Y.pestis* lipid A	*H.pylori* lipid A
			
Endotoxic Activity: **+++**	Endotoxic Activity: **++**	Endotoxic Activity: **++(?)**	Endotoxic Activity: **++**
P.aeruginosa lipid A	*C.trachomatis* lipid A	*B.fragilis* lipid A	*B.pertussis* lipid A
			
Endotoxic Activity: **+**	Endotoxic Activity: **+**	Endotoxic Activity: **+**	Endotoxic Activity: **+ (?)**
R.sphaeroides lipid A	*P. gingivalis* lipid A	Compound 406 (Ia)	Lipid X
			
Endotoxic Activity: **-** **(LPS antagonist)**	Endotoxic Activity: **+** (TLR-2 agonist)	Endotoxic Activity: **-** **(LPS antagonist)**	Endotoxic Activity: - (Very weak antagonist)

Figure 1.6 List of different Lipid A architectures and endotoxic activities (Erridge *et al*, 2002)

A list of structures of some Lipid A's from common organisms as well as some less common ones. The endotoxicity of the Lipid A's is also shown with the most endotoxic at the top left and the least endotoxic at the bottom right. (Erridge *et al*, 2002)

As can be seen in Figure 1.6, many of the organisms that have very high levels of endotoxicity are enterobacteria such as; *E. coli*, *Salmonella* spp and *Klebsiella pneumoniae*. However, there are also non-enterobacterial Lipid A's that exhibit high levels of activity e.g. *Haemophilus influenzae* or *Neisseria meningitidis*. (Netea *et al*, 2002; Erridge *et al*, 2002; Dehus *et al*, 2006; Bos *et al*, 2007).

It has been observed that Lipid A, isolated from other species can also vary greatly. The D-glucosamine in some species is replaced by 2,3-diamino-2,3-dideoxy-D-glucose; the number of acyl groups varies between 4,5,6 and 7 groups; the acyl groups chain lengths also vary; the symmetrical layout of the acyl groups (3+3, 4+2, 2+2) varies, as does the replacement of phosphate groups (Seydel *et al*, 2000; Brandenburg & Wiese, 2004; Fujimoto *et al*, 2005; Schromm *et al*, 2007). Low activity Lipid A moieties often have penta-acylated structures e.g. *Porphyromonas gingivalis*, *Rhodobacter sphaeroides*, *Bordetella pertussis* or the Lipid A precursor Ia (compound 406) (Erridge *et al*, 2002). In fact precursor Ia (compound 406) and *Rhodobacter sphaeroides* both have antagonistic actions. Certain hexa-acylated Lipid A species also have a low endotoxicity e.g. *Legionella pneumophila*, which in this case is due to an extended acyl group that is 18 carbon atoms in length. Another example is *Aquifex pyrophilus*, which is a hyperthermophile. This organism's LPS has a distinctive Lipid A type as it has no phosphate group. Instead it has a D-galacturonic acid linked to positions 1 and 4' instead (Alexander & Rietschel, 2001; Netea *et al*, 2002). Ancuta *et al*, (1996) isolated an LPS from *Francisella tularensis*, which has an inert Lipid A i.e.

it is neither an agonist stimulating immune response, nor is it an antagonist inhibiting other normal LPS types.

A lot of work has been done on the relationship between the biochemical composition of the Lipid A moiety and its three-dimensional shape and its biological activity (Seydal *et al*, 2000). Those LPS molecules that have a conical structure e.g. *E. coli*, have a much higher endotoxicity level, than those with a cylindrical conformation, such as precursor Ia (compound 406), *Rhodobacter capsulans* or *Chromobacter violaceum* (Seydel *et al*, 2000; Netea *et al*, 2002). Numerous factors enter into play, such as the nature, chain length, number and asymmetry of the acyl chains. In addition there is the number and distribution of negative charges, which governs the three-dimensional structure of Lipid A (Rietschel *et al*, 1994; Schromm *et al* 1999; Netea *et al*, 2002). This has led to the hypothesis that endotoxic activity is not the sole property of endotoxin, but that any molecule with such characteristics would also have endotoxic activity (Schromm *et al*, 1999).

1.2 The innate immune system and endotoxin

1.2.1 General overview of the innate immune system

The main functions of the innate immune system are the recognition of pathogens and providing a near instantaneous defence without the need for prior exposure, such as would be required with the acquired immune system (Vasselon & Detmers, 2002; Kaisho & Akira, 2006). Until reasonably recently the innate immune system was considered to be naive and simplistic, compared with the acquired immune system. This is known to be incorrect and a high level of complexity has now been shown (Triantafilou & Triantafilou, 2005).

There are three main parts to the innate immune system, the mechanical, the chemical and the cellular. The mechanical part of the immune system is made up of both physical barriers, such as the epidermis or the mucosal linings and physiological functions, which includes cilia action, desquamation and mucous discharge. The chemical part of the innate immune system is easily sub divided into three more categories. These are soluble or cell-linked pattern recognition receptors (PRRs), proteins or peptides with hydrolytic effects on micro-organisms and the cytokines and chemokines, which modulate and coordinate the innate immune response. The cellular part of the innate immune system contains a wide variety of host types, which include epithelial cells, mast cells, macrophages, neutrophils, natural killer cells and dendritic cells (Basset *et al*, 2003).

The main difference between the acquired and innate immune systems lies in the differences of the receptors of the two systems. The acquired immune system's T and B cell receptors are continually created throughout the development of the cells. The

receptors are created in such a way so as to have a unique receptor on each lymphocyte. The receptors are not kept encoded for ever nor are they passed on the next generation. There are believed to be between 10^{14} to 10^{18} different T and B cell receptors (Medzhitov & Janeway, 2000). The innate immune system receptors are found on a much wider variety of cells, such as macrophages, dendritic cells, certain specific T-cell types and also B-cells. They have also been isolated from non-immune cells like fibroblasts and epithelial cells (Medzhitov & Janeway, 2000; Medzhitov & Janeway, 2000a; Akira *et al*, 2006). The specificities of the receptors are not affected by which cell it is being expressed upon (Vivier & Malissen, 2005). The receptors also initiate an immediate effect. There is no wait for a proliferation process, as in the acquired immune system (Medzhitov and Janeway, 2000).

Structurally PRRs are made up of a wide variety of different families of proteins. These include leucine-rich repeat domains, calcium-dependent lectin domans and scavenger-receptor protein domains. There are three main methods for PRRs to act: secretion, endocytosis and signalling. An example of a secreted molecule is mannan-binding lectin; it acts by binding to specific carbohydrate structures on the surfaces of the micro-organisms. Once bound it is able increase the binding potential of the micro-organism and thus make it more susceptible to phagocytosis and the complement system. Endocytic PRRs are found on the surface of phagocytes are extremely important in distinguishing very specific highly conserved microbial structures, which then facilitates the uptake of the micro-organisms into lysosymes, where they are destroyed. The Toll-like receptor (TLR) family is an extremely important type of signalling receptor. It identifies specific highly conserved microbial structures and stimulates signal transduction cascades that lead to expression of a

wide variety of immune-response genes, such as inflammatory cytokines (Medzhitov and Janeway 2000).

Rather than trying to identify all the possible antigens that might be presented by micro-organisms, PRRs recognise a small number of highly conserved structures which are held in common across numerous microbial species (Akira *et al*, 2006). The highly conserved microbial structures are often referred to as pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 1998). Examples of common PAMPs found in micro-organisms includes, lipopolysaccharide, petidoglycan, lipoteichoic acids, mannans, bacterial DNA and double stranded RNA (Medzhitov and Janeway 2000a).

Whilst there are a wide variety of different chemical compositions of PAMPs, there are three characteristics common to all of them. Firstly, PAMPs are microbial structures not found within host cells or molecules. Secondly, they are vital either for the viability or the pathogenicity of the micro-organism. Thirdly, PAMPs from a given microbial class are normally found throughout that entire microbial class (Beutler, 2004; Akira *et al*, 2006; Horner, 2006). Being vital for the survival or pathogenicity of the microbe is a way to make sure that the mutation of the PAMPs is kept to a minimum, as any mutation would be likely to the disadvantage of the organism with respect to the un-mutated strains (Beutler, 2004).

1.2.2 The role of endotoxin

The outer membrane of Gram-negative bacteria consists of a variety of amphiphilic molecules; of these molecules lipopolysaccharide is normally considered to be the most important immunologically and microbiologically as the principal surface

antigen (Dixon & Darveau, 2005). Endotoxin acts as the main indicator to the immune system of the presence of Gram-negative bacteria (Medzhitov & Janeway, 2002).

Endotoxin has two main roles in the outer membrane, firstly it acts structurally as a supportive macromolecular domain of the cell envelope and secondly it acts functionally as a selective permeability barrier to molecules of either negatively charged or hydrophobic domains, due to its highly anionic nature (Horn *et al*, 1996; Lerouge & Vanderleyden 2002). It is also thought to be an adsorption receptor for certain bacteriophages. There is also the toxicity and immunongenicity to a wide variety of higher organisms (Leive *et al*, 1968; Morrison & Leive, 1975).

It has been known for a long time that pure endotoxin is extremely toxic if injected systemically and has very similar physiological effects to Gram-negative bacteraemia (Rietschal *et al*, 1994; Horn *et al*, 1996). It is due to this that endotoxin is considered to act as the main molecule that informs the immune system of the presence of Gram-negative bacteria in the body (Horn *et al*, 2000)

It has been shown that endotoxic recognition is mediated by specific host receptors (Schletter *et al*, 1995; Beutler & Poltorak, 2001). This allows even very low concentrations of endotoxin to stimulate a large immune response. Since the host receptors act as signal amplifiers, the immune response is boosted beyond what would be normal for the immune response to that concentration of another molecule (Beutler & Rietschel, 2003). Endotoxin acts almost solely through its Lipid A moiety, through the stimulation of a potent inflammatory response, which is entirely conferred by the host (Riestchel *et al*, 1994; Beutler & Rietschel, 2003). There are other bacterial structures that have endotoxic-like activity, such as lipopeptides, peptidoglycans,

lipoteichoic acids, double stranded RNA and some bacterial exotoxins. In the majority of cases the outcome is a successful resolution of the bacterial infestation, rather than the development of lethal toxicity (Beutler & Rietschel, 2003).

Macrophages are the most important cell involved in the immune response to endotoxin (Michalek *et al*, 1980; Freudenberg *et al*, 1986). This is because they are the main source of tumour necrosis factor alpha (TNF-α), which is a potent pro-inflammatory cytokine (Mannel *et al*, 1980). It is possible though for endotoxin to stimulate other cells, such as endothelial cells, smooth muscle cells and neutrophils to produce and release other mediators of an endogenous origin. These mediators include bioactive lipids (e.g. platelet activating factor or thromboxane A2), reactive oxygen species (e.g. nitric oxide) and most importantly cytokine proteins such as interleukin-1 (IL-1), IL-6 and TNF-α. The number of endotoxin molecules present is directly proportional to the amount of mediators released, up to a threshold, which in turn is proportional to the pathophysiological reaction to the endotoxin (Schletter *et al*, 1995; Beutler & Poltorak 2001). TNF-α is considered to be one the most important contributors to the lethal aspects of endotoxin exposure and thus its production is an excellent measure of endotoxic activity with the immune system. It is for this reason that the macrophages are considered one of the best targets for endotoxic research (Beutler *et al*, 1985; Poltorak *et al*, 2000).

Endotoxin interaction with the immune system is a constantly changing dialogue. There are a variety of mechanisms deployed by the host to deal with endotoxin. Thus to understand endotoxic interactions all the different mechanisms have to be considered together, as there is a large amount of overlap between the different

mechanisms. So it is impossible to consider one mechanism without viewing the whole (Elsbach, 2000).

The mechanisms of interaction between endotoxin are both intracellular and extracellular; these act in concert to stimulate the immune system. The extracellular mechanism consists mostly of recognition molecules that bind to the endotoxin and signal the intracellular mechanisms which stimulate the production of an immune response (Antal-Szalmás, 2000).

1.2.3 Innate immune recognition of endotoxin

This process is summarised in Figure 1.7 below.

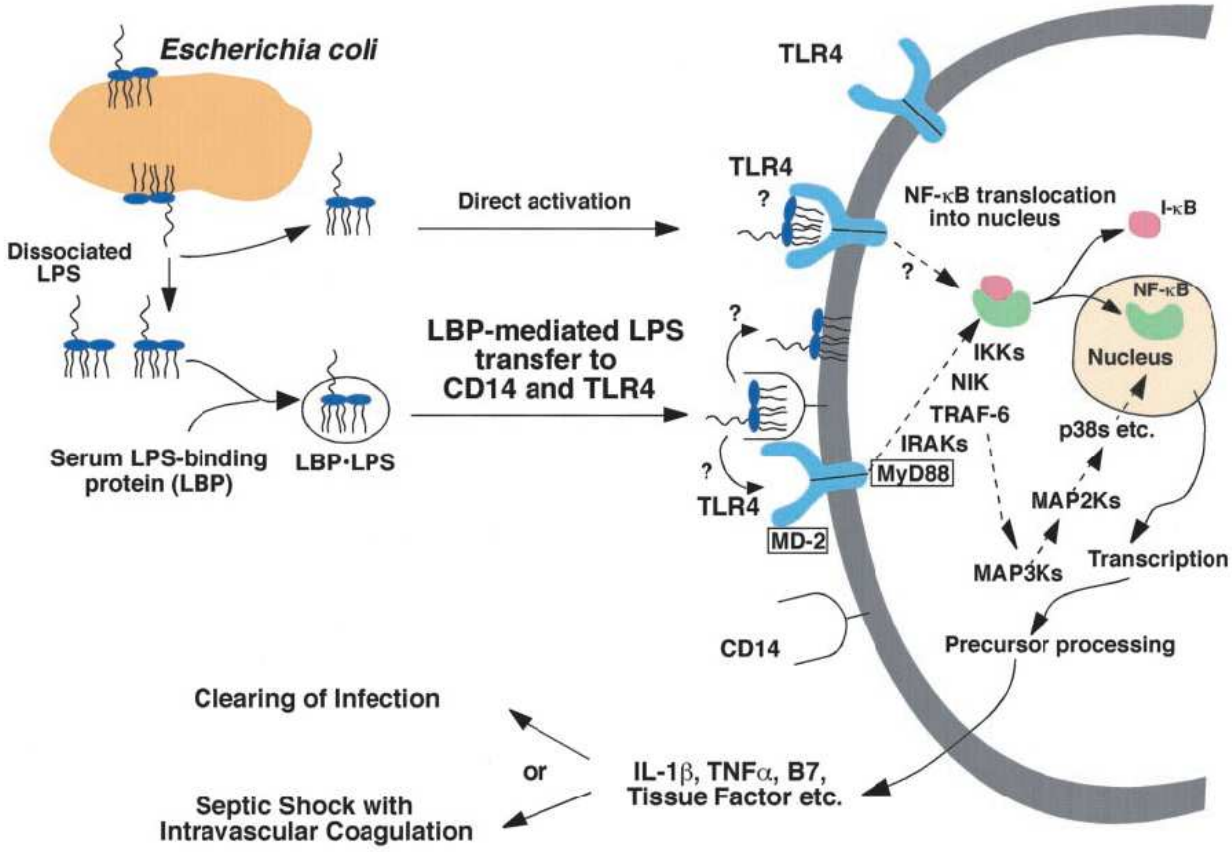


Figure 1.7: Recognition of endotoxin by the innate immune receptors.LPS acts through a variety of receptors, but most importantly through TLR-4 with mediation from LBP, CD-14 and MD-2 (Raetz & Whitfield, 2002).

1.2.3.1 Lipopolysaccharide binding protein (LBP)

LBP is the thought to be the first host receptor to interact with the LPS and to start the inflammatory response (Schumann *et al*, 1990).

LBP is a 50 kDa polypeptide normally produced in hepatocytes, where it is released as a glycoprotein into the blood stream (Schumann *et al*, 1990). Other cells have also been shown to produce LBP including; epithelial cells of the skin, the lung, the intestine, as well as human gingival tissues, lung arteries, heart muscle cells and renal cells (Su *et al*, 1994; Dentener *et al*, 2000).

LBP's primary function is to act as an amplifying system that is capable of alerting immune cells to tiny quantaties of LPS present (Elsbach, 2000).

LBP is normally found in the human serum at a concentration of between 5-15µg/ml (Zweigner *et al*, 2001). The concentration of LBP increases 50-100 fold in inflammation due to either increased production caused by the inflammatory response or caused by the presence of microbial stimuli (Tobias *et al*, 1999).

At physiological concentrations, LBP greatly enhances the close contact between LPS and other recognition molecules. Either those in serum, like soluble cluster of differentiation (CD) -14 and high density lipoprotein or those attached to cells, such as membrane CD-14 (Freudenberg *et al*, 2001). These interactions can bring about the inhibition of LPS by high density lipoprotein or activation of cells by CD-14. This

means that the host response is governed by which of the two processes is faster. Studies into the kinetics of the two reactions have shown that LPS/LBP complexes faster with CD-14. This suggests that an initial immune cell activation is beneficial, but the cell activation is neutralised by the action of high density lipoprotein to stop the overstimulation of the response (Heumann and Roger, 2002).

LBP has been shown to increase the endotoxin-mediated stimulation of CD-14-positive cells by 100-1000 fold in a serum free media. It has also been shown that LBP transfers LPS to soluble CD-14 (sCD-14) and this is able to activate membrane CD-14 (mCD-14) deficient cells, such as endothelial and epithelial cells (Zweigner *et al*, 2006). LBP acts as an integral part of the stable trimolecular complexes, where it is able to interact with LPS and CD-14. This allows monocytes to respond to concentrations as low as 10pg/ml (Thomas *et al*, 2002). It has also been suggested that LBP is able to present hundreds of LPS molecules to CD-14 without itself being consumed (Tobias *et al*, 1995).

The reduction or removal of LBP in laboratory animals brings about almost the complete removal on any LPS–induced toxicity, which indicates the importance of LBP for transfer of LPS to the receptor complex *in vivo*. Many studies have shown that LBP deficient mice are unable to generate a successful immune response to Gram-negative bacteria, such as *Salmonella typhimurium*, *Klebsiella pneumonia* and *E. coli* and suffered increased mortatily due to bacterial over growth (Opal *et al*, 1999; Branger *et al*, 2005).

1.2.3.2 CD-14

CD-14 is likely to be the important second step in the LPS-signalling pathway, as once the LBP-LPS complex is completed it is bound to either sCD-14 or mCD-14, which in turn leads to activation of the immune cells (Dixon & Darveau, 2005).

CD-14 deficient cell lines are unresponsive to LPS, but regain their ability to sense LPS when transfected with CD-14 (Lee *et al*, 1992). Genetically CD-14-deficient human monocytes have very low affinity for LPS binding (Couturier, 1991).

The formation of the CD-14 - LPS complex decreases the concentration of LPS required to mediate signalling by 100-1000 fold (Landmann *et al*, 2000). CD-14 doesn't have identical binding affinity for all LPS types. It has been shown by Cunningham *et al*, (1999) that *E. coli*, *Helicobacter pylori* and *Porphyromonas gingivalis* LPSs bind in different ways to CD-14. The amount of LPS from *Porphyromonas gingivalis* required to gain a 50% bind to CD-14 was ten times that of *E. coli* LPS.

CD-14 seems to be able to measure the concentration of LPS and in response to this to change the charge on its surface, which promotes LPS interactions in an electrostatic manner (Wright, 1995). This carrier role of CD-14 is unable to distinguish between different microbial ligands (Wright, 1995; Cunningham *et al*, 1999). It seems instead to be a step to collect sufficient LPS for recognition by a second receptor (Funda *et al*, 2001).

CD-14 is also able to bind to other microbial structures, which suggests that CD-14 is a possible PRR (Freudenberg *et al*, 2001). These structures include peptidoglycan from Gram-positive cell wall and *Porphyromonas gingivalis* fimbrie (Pugin *et al*,

1994; Hajishengallis *et al*, 2006). This ability to bind to a variety of PAMPs from different organisms might be the reason why sepsis is so similar whether caused by Gram-negative, Gram-positive or even fungi (Landmann *et al*, 2000).

1.2.3.3 Myeloid differentiation protein 2 (MD2)

MD2 is a small cysteine rich glycoprotein, which is secreted from B-cells (Janssens & Beyaert, 2003). It is known to join with the ectodomain of toll like receptor 4 (TLR4). It has also been shown that TLR4 is unable to transduce LPS signalling without the attachment of MD2 (Shimazu *et al*, 1999). If the MD2 gene expression is disrupted in mice then it halts LPS signalling, showing MD2's importance in TLR4 function (Schromm *et al*, 2001; Nagai *et al* 2002). Some putative methods of MD2, TLR4 interaction are suggested in Figure 1.8.

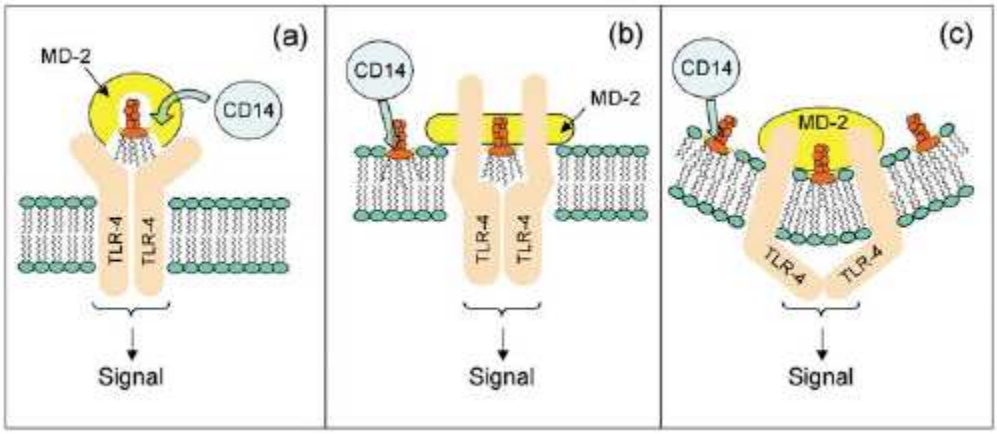


Figure 1.8: Possible methods of CD-14/LPS binding to the TLR4 and MD2 complex. (a) CD-14 delivers the LPS directly to the MD2/TLR4 complex inducing dimerisation. (b) CD-14 catalyses insertion of the LPS into the cell membrane, where the hydrophobic domain interacts with the transmembrane domain of TLR4 and the head region interacts with the MD2. (c) insertion of the LPS into the cell membrane causes changes in the membrane architecture sensed by TLR4/MD2 complex (Erridge *et al*, 2002)

1.2.3.4 Toll like receptors (TLR)

TLRs are a family of PRR which is made up of type 1 integral transmembrane glycoproteins. These contain leucine rich repeat (LRR) units in the pathogen binding ectodomains (ECD). Signal transfer occurs due to intracellular structural components. This includes an almost identical structure in both TLR and IL-1 receptor family members, known as Toll/IL-1 receptor homologous (TIR) domain, which represents the cytoplasmic end (Akira *et al*, 2006; Kaisho & Akira 2006; Pandey & Agrawal, 2006). This TIR domain has been found in a wide variety of transmembrane and cytoplasmic proteins in plants, worms, arthropods, and even some bacteria. All these molecules are involved in host defence, thus making TIR one of the earliest signalling domains found (Aravind *et al*, 1999).

So far 13 mammalian TLRs have been recognised; 11 of these are expressed in humans, TLR 1-11. Each TLR is activated by a variety of different PAMPs, sometimes from a large number of different organisms. The different PAMPs each TLR is activated by are shown in Figure 1.9.

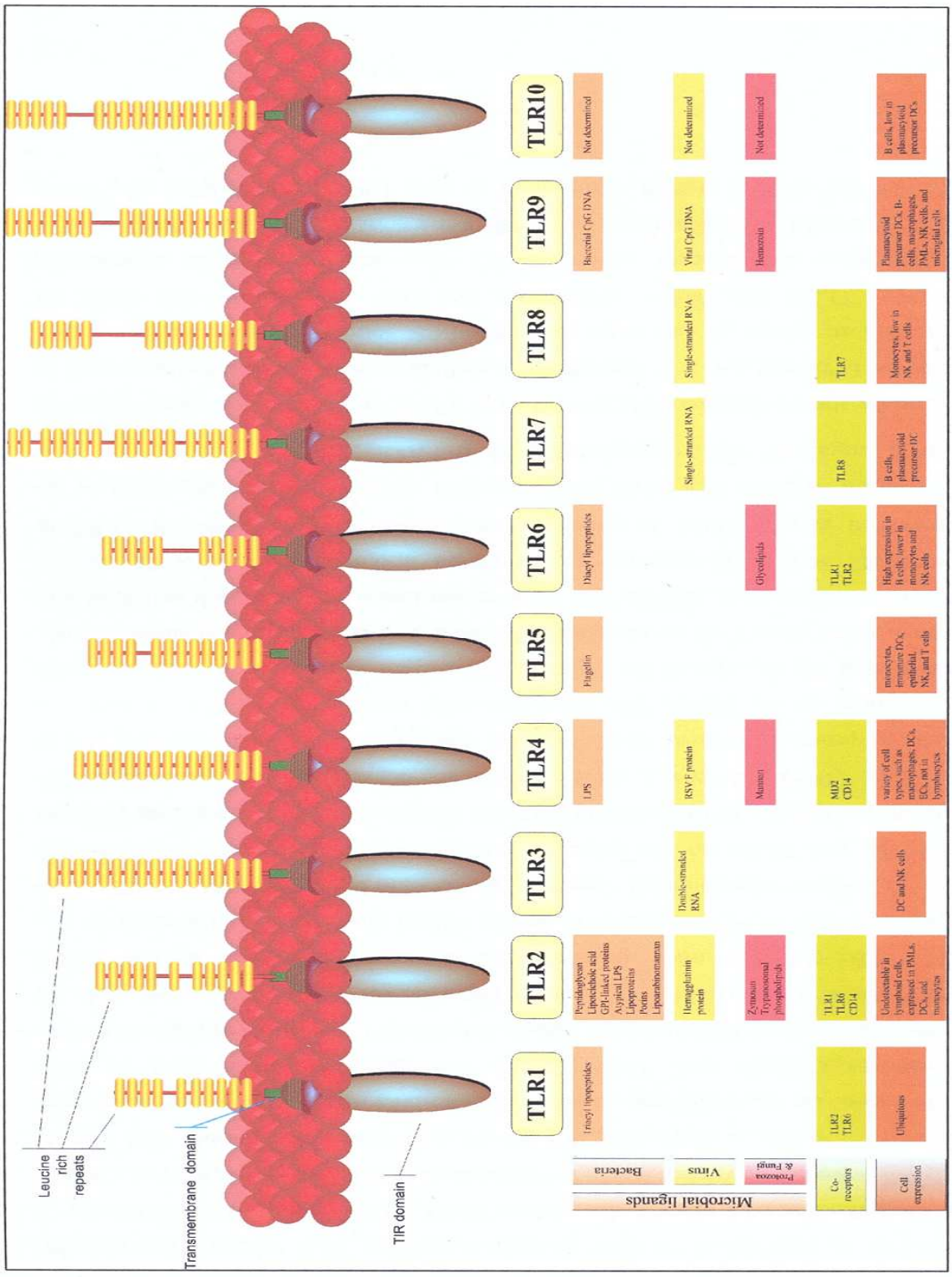


Figure 1.9 TLR ligand Specificities. A list of the different TLRs and the variety of ligands they can bind to. (Janssens & Beyaert 2003)

Of these receptors, the ones of most interest in the investigation are TLR2 and TLR4 due to their relation with endotoxin (Schaub *et al*, 2006)

TLR2

TLR2 has the ability to recognise PAMPs from a wide range of micro-organisms, including Gram-positive bacteria, Gram-negative bacteria, mycobacteria, fungi, parasites and viruses (Raetz & Whitfield, 2002; Texereau *et al*, 2006).

TLR2 has the ability to bind to LPS and activate an inflammatory immune response. It is still undecided as to how important a receptor for LPS it is (Hirschfield *et al*, 2000). It has been shown that some of the expression to LPS was due to endotoxic proteins attached to the LPS, as when the LPS was re-extracted to remove the protein, signalling through TLR2 stopped (Brightbill *et al*, 1999; Hirschfeld *et al*, 1999; Hirschfeld *et al*, 2000). The above results were carried out with Enterobacteriaceae. TLR2 is well documented to be able to bind and signal LPS structures from other bacterial species, such as *Leptospira interrogans*, *Porphyromonas gingivalis*, *Helicobacter pylori*, *Rhizobium* species Sin-1, *Legionella pneumophilia*, *Bacteroides fragilis* and *Pseudomonas aeruginosa* PAC-611 (Hirschfeld *et al*, 2001; Werts *et al*, 2001; Smith *et al*, 2003; Girad *et al*, 2003; Erridge *et al*, 2004). These organisms tend to have different structural forms from those of classic LPS, such as that of *E. coli*, which activates TLR4. The variations including the different distribution and differentiation of acyl chains in the lipid A moiety seems to have a different recognition style (Netea *et al*, 2002). The conical and cylindrical types of LPS may bind to and signal through different receptor complexes. This was shown by the fact that *Porphyromonas ginigivalis*, which has a cylindrical LPS signals through TLR2, whilst the conical LPS of *E. coli* signals through TLR4 (Hirschfeld *et al*, 2001; Netea *et al*, 2002). So it seems that TLR2 signals the less classically shaped LPSs.

TLR4

TLR4 is considered to be the main receptor for LPS signalling in the innate immune system. The main pieces of evidence to support this are: firstly the loss of the LPS response due to a specific mutation in *lps* locus in a mouse model, this *lps* locus is identical to the *tlr4* locus and they both encode TLR4, secondly re-purified LPS was unable to elucidate a response from a non-responder mouse strain C3H/HeJ, thirdly no human or murine TLR2 transfected cells showed any response to either re-purified LPS or protein- free synthetic Lipid A and finally the unresponsiveness of these TLR4 deficient mice when challenged with lethal dose of purified LPS (Poltorak *et al*, 1998; Hoshino *et al*, 1999; Hirschfeld *et al*, 2000; Heumann & Roger, 2002).

The biophysical properties of LPS molecules have been suggested to have a role in TLR signalling. It is thought that the hexa-acyl conical structure of the Enterobacteriaceae LPS is more favourable to signalling through TLR4, whilst the penta-acyl cylindrical structure LPS is more able to signal through TLR2 (Schromm *et al*, 2000; Netea *et al*, 2002). Tetra-acyl Lipid A alone is unable to stimulate human TLR4, whilst hexa-acyl Lipid A does (Poltorak *et al*, 2000). It has been suggested that human TLR4 is able to read the LPS structure, thus evaluating the nature of the acyl chains, allowing the differentiation between Lipid A and tetra-acyl Lipid A (Poltorak *et al*, 2000). The other suggestion is that the TLR4 is able to distinguish between the different conformations of the CD-14 molecules when they are bound to the different Lipid A structures (Lien *et al*, 2000; Poltorak *et al*, 2000).

A reason that TLR4 is considered to be the main receptor for LPS could be that the vast majority of studies into TLR signalling used only classical LPS structures. These

were mostly *E. coli* and *Salmonella* spp.. More than 90% of all endotoxin publications were done with LPS from Enterobacteriaceae (Dehus *et al*, 2006).

TLR4 also is able to signal PAMPs from other organisms, such as a specific viral protein from Hepatitis C, plant paclitaxel and the fusion protein from respiratory sysncytial virus (Kurt-Jones *et al*, 2000; Akira *et al*, 2006; Machida *et al*, 2006).

TLR4 is able to induce a number of different immune responses through their cytoplasmic signalling domains. It has a very strong similarity to IL-1 receptor (IL-1R). Myeloid differentiation factor 88 is recruited to the activated sites on TLR cystolic domains. From here it is able to catalase the activation of the serine/threonine kinases of the IL-1 receptor-associated kinases (IRAK) family. The IRAK family then acts through TNF-receptor associated factor 6 (TRAF-6) to promote myeloid-activated protein (MAP) kinase cascades and the nuclear factor (NF)-κB-inducing cascade (Underhill & Ozinsky, 2002). This leads to the production of a variety of pro-inflammatory cytokines, most notably TNF-α, INF-γ, IL-1β and IL-6, as well as chemokines IL-8. These chemicals lead to development of fever and also up-regulate the acute response through the production of complement, C-reactive protein and other molecules (Basset *et al*, 2003).

1.3 Hospital Acquired Pneumonia (HAP)

1.3.1 The disease

HAP or nosocomial pneumonia is a case of pneumonia acquired whilst in a nosocomial environment. It is generally defined as an inflammation in the substance of the lungs, brought about by a microbial infection of the lower respiratory tract. The infection is most often caused by bacteria, but fungal pneumonia has also been isolated. Viral pneumonia is rarely seen in adults, although it is a slightly more common in children, where it is normally accompanied by a bacterial infection (Kumar & Clark, 2002; Langley & Bradley, 2005).

Patients that develop pneumonia whilst in hospital are usually immunocompromised, mostly due to an underlying condition. This allows the micro-organisms to gain access to the lungs, as the immune system is too weak to remove the infections. Other risk factors for the development of HAP include being attached to a ventilator, which inhibits the cough reflex, the bacteria are also able to colonise the tubes with biofilms. This means that the patient is continuously breathing in bacteria. There is also a greater prevalence of antibiotic resistant organisms in hospitals than in the community, which mean that it is harder to eradicate the infection with antibiotic therapies. Recovering from surgery is another major risk for patients as the immune system is already under pressure and can easily become over stretched, allowing the micro-organism to gain a foothold in the lung (Berba *et al*, 1999; Mehta & Niederman, 2003; Wolkewitz *et al*, 2008).

1.3.2 Incidence and Epidemiology

HAP is a huge problem in the intensive care units (ICUs) of nearly all major hospitals. HAP is the second most common type of nosocomial infection and accounts for a quarter of all infections in the ICU. When combined with primary bacteraemia, HAP is the leading cause of death from infection (Bowton, 1999; Beardsley *et al*, 2006). The rate of HAP varies dramatically between different hospitals. This can be seen by the wide variations measured by the different percentages of incidences of HAP. These can vary between 9% and 68%, depending on the hospital (David & Bowton, 2006). In a study of 98 hospitals around the world, Rosenthal *et al* (2008) recorded the incidence of ventilator associated pneumonia (VAP) in patients on mechanical ventilators across different ICU types. These varied from 40.74% of patients in medical ICUs (2408 patients), 19.82% in medical surgery ICUs (26155 patients), to 7.85% in paediatric ICUs (1808 patients). So it can be seen from this data that HAP is indeed a global problem.

The main difference between community acquired pneumonia (CAP) and HAP is the causative organisms. In CAP the main organism is *Streptococcus pneumoniae*, with 50% of cases caused by this organism. *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and Influenza virus account for 5% each of the cases (Kumar & Clark, 2002). In HAP the microbiology is very different with a variety of less directly pathogenic species causing the infections due to antibiotic resistance. A study of the micro-organisms isolated from patients in post surgical ICU wards from hospitals across France was carried out by Dupont *et al* (2003). These are displayed in table 1.1 below.

Table 1.1 The Bacteriology of Postoperative Pneumonia EOLE study.

Organism	Number of Isolates	Percentage of total
E. coli	46	9%
Klebsiella pneumoniae	12	2.4%
Enterobacter spp.	12	2.4%
Serratia spp.	15	3%
Pseudomonas aeruginosa	56	11%
Acineobacter baumannii	6	1.2%
Haemophilus influenzae	63	12%
Morganella spp.	17	3.4%
Miscelleanceous Gram-negative bacteria	80	16%
Coagulase positive *Staphylococcus* spp.	88	17%
Coagulase negative *Staphylococcus*	22	4.4%
Streptococcus pneumoniae	31	6.1%
Other S*treptococcus* spp.	34	6.7%
Yeasts	23	4.6%

As can been seen in table1.1, the incidence of organisms is very different from that displayed by CAP infections. *Streptococcus pneumoniae* is also found on the list but there was only an incidence of 6.1% in the study compared to an incidence of 50% in CAP infections. The most common organisms in HAP are coagulase positive *Staphylococcus*, most often *Staphylococcus aureus.* This is due to their innate pathogenicity, as well as their being resistant to a wide variety of antibiotics. The most well known of these resistant staphylococci is methicillin resistant

Staphylococcus aureus (MRSA), which is likely to be the main organism in the coagulase positive *Staphylococcus group* (Wolkewitz *et al*, 2008).

With regard to Gram-negative organisms, there is a much greater range of causative organisms, with the *Enterobacteriaceae* as a group being the most prevalent but the *Haemophilus influenzae* being the most common Gram-negative organism. *Haemophilus influenzae* is an organism that can cause CAP but it is much rarer than *Streptococcus pneumoniae* (Kumar & Clark, 2002; Dupont *et al*, 2003). It is an opportunistic pathogen that often causes lower respiratory tract infections. It is commonly found in the upper respiratory tract as a commensal but it is able to infect an immunocompromised patient, given the opportunity (Jorgensen *et al*, 1990; Gür *et al*, 2002).

Pseudomonas aeruginosa, the second most commonly isolated Gram-negative bacterium, is an organism that only rarely causes CAP. It is a common environmental organism found throughout nature in soil and water samples. It is not in itself particularly pathogenic but it is a quintessentially opportunistic pathogen able to infect people, whose immune systems are rundown and no longer functioning fully. It is also resistant to a wide variety of antibiotics, which means that it is able to survive in a hospital environment without much difficulty (Kollef *et al*, 2005; McGowan, 2006)

Members of the *Enterobacteriaceae* are normally found in the gut but when a patient is immunocompromised they are able to translocate through the gut wall and thus spread throughout the body to cause a wide variety of diseases. Pneumonia is a common side effect of this and is a major problem. Again there is a wide level of

antibiotic resistance in these organisms, allowing them to colonise in a hospital environment (Kollef *et al*, 2005; Alekshun & Levy, 2006).

1.3.3 Treatment regimes

There are a number of different antibiotics available to physicians treating patients with HAP. Which one is to be used depends very much on the organism that is present and also the particular resistances the organism might show. This is why it is crucial to get samples sent to the laboratory for testing quickly. It is also important for accurate results to be returned swiftly so that the correct drug can be given (Kumar & Clark, 2002).

There are a number of difficulties with correctly isolating and identifying the causative organism though, as the samples taken from the lung to be tested for bacterial presence are usually sputum. This can be extremely difficult to isolate bacteria from, as the bacteria can often form biofilms in the sputum which makes them harder to isolate on agar. The sputum is normally isolated from the upper airways and thus is not representative of the lower respiratory tract and so could give incorrect results for the microbiological cause of the pneumonia. Sputum is also often contaminated with a variety of commensal bacteria, from the mouth and the upper respiratory tract, which may lead to incorrect identifications. Some organisms are more effective growers than others and so even though they may not be the causative organism they may appear to be in the laboratory. *P. aeruginosa* is a good example of this, as it is a very effective grower much more so than something like *Haemophilus influenzae* and on normal agar will grow easily whilst *Haemophilus influenzae* will be unable to compete. The use of bronchial lavages is a possibility to improve the accuracy of the results as these get bacteria from the lower respiratory tract, but they

can be dangerous to perform with severely ill patients. Proper use of selective media will aid with the removal of false positive results (Berba *et al*, 1999; Kollef *et al*, 2005; Wolkewitz *et al*, 2008)

Initial treatment regimes, before the results from the lab are available, should be tailored to the make up of local microbiological flora. This will reduce the use of ineffective antibiotics and reduce resistance forming (Beardsley *et al*, 2006).

The fact that the majority of people suffering from HAP have another underlying condition must be taken into consideration when designing an antibiotic regime. This condition may already require an aggressive regime of antibiotics and so any new ones will have to be added in carefully to avoid exacerbating the original condition (Kumar & Clark 2002).

The rise of antibiotic resistance in the last 30 years is leading to a great deal of problems. The rise of MRSA has already meant that very few antibiotics can be used to treat this organism. There are now also pan-resistant strains of other bacteria species now found in a hospital environment, such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* (Trouillet *et al* 1998; Giamarellou *et al*, 2008). This means that there are no possible treatments for people infected with these organisms. As a result, it is essential that alternative non antibiotic treatments for people suffering from HAP are developed, such as passive vaccines or cationic peptides (Vos *et al*, 2005).

1.4 *Acinetobacter calcoaceticus- Acinetobacter baumannii*

1.4.1 *Acinetobacter calcoaceticus- Acinetobacter baumannii* biology and taxonomy

Acinetobacter calcoaceticus- Acinetobacter baumannii are Gram-negative coccobacilli, which are known to be non-fermentative, non-motile, non-pigmented, catalase-positive and oxidase-negative. They are usually found occurring in diplococcal formations, but can be found in chains of varying lengths (Giamarellou *et al*, 2008). Traditionally the species was considered to have been a commensal with low pathogenicity and even used to be ignored when it was isolated from clinical specimens in the 1970s (Bérgogne-Bérèzin & Towner, 1996). However within the last 20 years interest has grown dramatically in these organisms, especially in *Acinetobacter baumannii*, for two main reasons. Firstly the proliferation of ICUs has brought about a dramatic change in the type of infections caused by *Acinetobacter baumannii* and secondly the emergence of multidrug-resistant strains some of which are resistant to all known antibiotics, with the exception of colistin (Rello & Diaz, 2003; Giamarellou, 2006; McGowan, 2006; Giamarellou *et al*, 2008). It has even been suggested that we are closer to the end of effective antibiotic therapy with *Acinetobacter baumannii* than with MRSA (Hanlon, 2005)

The taxonomy of *Acinetobacter* spp. is confusing and the organism has had a variety of classifications, such as in the families Neisseriaceace or Moraxellaceace. It has also had a variety of names within these families, such as *Moraxella*, *Herellea*, *Achromobacter* or *Alcaligenes* (Hanlon, 2005; Giamarllou *et al*, 2008). The situation is still not fully resolved and *Acinetobacter* spp. are undergoing continuous changes

of nomenclature. So far 32 genomic species have been identified of which 17 have been given names (Van Looveren and Goosens, 2004). Traditional identification methods have been found to be generally ineffective, such as *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, along with the as yet unnamed genospecies 3 and 13 sensu Tjernberg and Ursing (TU), are very hard to distinguish phenotypically and thus are often referred to as the *Acinetobacter calcoaceticus- Acinetobacter baumannii* complex (Tjernberg & Ursing, 1989; Dolzani *et al*, 1995). These strains are the most commonly associated with hospital acquired infections and they account for about 75% of *Acinetobacter* spp. isolated from clinical specimens (Henwood *et al*, 2002).

1.4.2 Epidemiology of *Acinetobacter calcoaceticus- Acinetobacter baumannii* complex

Acinetobacter calcoaceticus- Acinetobacter baumannii complex has been found in soil, water, animals and humans. The *Acinetobacter* spp. are normal inhabitants of human skin but have also often been isolated in the respiratory tract of hospitalised patients (Paterson, 2006; Fournier & Richet 2006). *Acinetobacter calcoaceticus* is a common environmental organism found ubiquitously throughout nature, but it has also been isolated from human clinical cases (Dijkshoorn *et al*, 2007). *Acinetobacter* spp. have also been extracted from some unlikely sources, such as food and arthropods. It has further been shown that *Acinetobacter* spp. can be found in about 17% of fresh fruit and vegetables. Of these *Acinetobacter baumannii* was isolated in 56% of isolated strains. The strains were isolated from a variety of common fruits and vegetables, such as apples, melons, beans, cabbage, cauliflower, carrots, potatoes, radishes and peppers (Berlau *et al*, 1999). It has been suggested that hospital food

could be a potential source of *Acinetobacter baumannii* infections, but since the preponderance of cases are in the ICU, this would seem to invalidate such a hypothesis (Corbella *et al*, 1996).

Acinetobacter spp. are common commensals of the human skin with up to 75% carriage in hospitalised patients and between 25% - 43% in the healthy population, although the majority of these were not the strains that are generally associated with nosocomial infections (Seifert *et al*, 1997; Sebeny *et al*, 2008). Even with this high commensal carriage of *Acinetobacter* spp., skin and soft-tissue infections are rare. In a study in America it was found that only 4.1% of all soft tissue infections were caused by *Acinetobacter baumannii* (Sader *et al*, 2002). There is never the less a large increase in soft tissue infections in traumatic injury patients, especially in patients in military hospitals in Afghanistan and Iraq (CDC, 2004; Fournier & Richet 2006; Sebeny *et al*, 2008). This was also observed in the Vietnam War, where *Acinetobacter baumannii* was the most commonly isolated organism from traumatic injuries (Fournier & Richet 2006).

Acinetobacter baumannii is most commonly associated with nosocomial pneumonia and bacteraemia, as are *Acinetobacter* genospecies 3 & 13 TU. Their ability to survive in hospital environments is mostly due to its resistance to antibiotics as well as its ability to survive in moist and dry environments (Giamarellou *et al*, 2008). This resistance to desiccation means that *Acinetobacter baumannii* can colonise many areas of hospitals, such as ventilators, mattresses, pillows, bed rails, bedsides, distilled water containers, intravenous nutrition equipment, drinking water, sinks, hygroscopic bandages, showers, stainless-steel trolleys, resuscitation equipment and tables, thermometers and soap dispensers (Paterson, 2006). So it can be seen that

Acinetobacter baumannii and *Acinetobacter* genospecies 3 & 13 TU are organisms that can be isolated from many areas in the hospital. It is due to these properties that *Acinetobacter baumannii* is such a huge problem in hospitals causing outbreaks. Most important though are their resistance to antibiotics. There are some strains that are pan-resistant to all known antibiotics, with the sole exception of colistin (Giamarellou, 2006; Petrosillo *et al*, 2008). This means that even though *Acinetobacter baumannii* is not as aggressive as other pathogenic species, in immunocompromised patients who are unable to fight off the infection themselves, it is unable to be treated effectively. This leads to outbreaks spreading through hospital wards especially the ICU and being unable to be contained with conventional treatments (Falagas *et al*, 2008).

There are a number of epidemic clones isolated so far. The three European ones are called clone I, II, and III. Clones I and II have caused outbreaks in north western Europe, with clone I also being found in Spain, South Africa, the Czech Republic, Poland and Italy, whilst clone II has also been isolated in Spain, South Africa, France, Greece and Turkey. Clone III has been isolated in France, Italy, Spain and the Netherlands. This suggests that these clones are highly virulent and also multidrug resistant. These strains cause outbreaks that are extremely difficult to control and can lead to endemicity of these strains in hospitals (Van Dessel *et al*, 2004).

1.4.3 *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex speciation

The *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex is a group of 4 very closely related organisms of *Acinetobacter calcoaceticus*, *Acinetobacter baumannii* and genospecies 3 and 13 TU. These organisms are almost impossible to

differentiate from each other by traditional phenotypic methods and it requires 16S - 23S DNA - hybridisation to differentiate between them (Tjernberg & Ursing, 1989, Dolzani *et al*, 1995). There is another method for the identification of *Acinetobacter baumannii* from the other organisms of the *Acinetobacter calcoaceticus* - *Acinetobacter baumannii* complex, because it has an OXA-51-like beta lactamase. This carbapenenmase is not found in the other organisms of the complex. *Acinetobacter* genospecies 13 TU on the other hand though can be differentiated from *Acinetobacter baumannii* through a PCR method that differentiates between differences in the *gyrB* gene sequences (Higgins *et al*, 2007).

Acinetobacter calcoaceticus is a common environmental organism that has been isolated from a wide variety of different of environments, such as soil, water, fruit and also humans. *Acinetobacter baumannii*'s natural reservoir has never been properly isolated, but it is now commonly isolated in nosocomial infections and from the skin. *Acinetobacter* genospecies 3TU has been isolated from humans, including clinical isolates, soil and fruit samples. *Acinetobacter* genospecies 13T U has been most commonly isolated from humans, including clinical isolates (Dijkshoorn *et al*, 2007).

This difficulty of being able to differentiate between the species of the complex has meant that there is high probability that there have been a number of mis-characterisations of organisms. So many organisms that have been thought of as *Acinetobacter baumannii* were in fact *Acinetobacter calcoaceticus*. This is likely to be the reason that many papers state that *Acinetobacter baumannii* is an environmental organism, whilst is highly unlikely that they are in fact *Acinetobacter baumannii*. This also carries over to the genospecies 3 and 13 TU as these can be mis-characterised as

Acinetobacter baumannii isolates, when they are not infact (Van Looveren *et al*, 2004).

1.4.4 *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex virulence factors

Whilst normally antibiotic resistance mechanisms are not considered virulence factors in the case of *Acinetobacter calcoaceticus - Acinetobacter baumannii*, but due to the very small number of virulence factors isolated from the organism so far and the fact that without these resistance mechanisms it would not be a hospital acquired infection. So it seems that in this case resistance mechanisms should be considered under the heading of virulence factors.

1.4.4.1 *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex lipopolysaccharide

Acinetobacter baumannii - Acinetobacter calcoaceticus complex LPS is one of the virulence factors exhibited by the organism. It is normally found in a smooth formation with a long O-polysaccharide chain. It is generally considered to be similar in activity to *E. coli* (Haseley *et al*, 1998; Vinogadov *et al*, 2003). *Acinetobacter baumannii - Acinetobacter calcoaceticus* LPS has been shown to signal the innate immune system through the TLR4, which indicates that it is similar in structure of its Lipid A moiety to the classical *E. coli* model (Erridge *et al*, 2007). LPS stimulation of the immune system has been suggested as the main cause of *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex disease (Leung *et al*, 2006).

1.4.4.2 Resistance mechanisms

Acinetobacter baumannii - Acinetobacter calcoaceticus complex has a wide variety of different resistance mechanisms which protect the organism from a large number of different antibiotics.

β-lactamases

These include β-lactamases, such as chromosomal cephalosporinases (AmpC), as well as a suggested novel family of β-lactamases, the *Acinetobacter* derived cephalosporinases (Corvec *et al*, 2003; Hujer *et al* 2005). Class A β-lactamases have been isolated such as TEM-1 and other extended spectrum β-lactamases (ESBLs), such as PER-1, SHV-12, TEM-92 and CTX-M-2 have also been isolated from a number of strains across the world (Poirel *et al*, 1999; Yong *et al*, 2003; Nagano *et al*, 2004; Perez *et al*, 2007).

Metallo-β-lactamases, such as IMP-1, IMP-2, IMP-4,IMP-5, IMP-6 and IMP-11, as well as VIM-2, have been isolated from a number of strains in Japan and Korea (Lee *et al*, 2004; Walsh *et al*, 2005; Perez *et al*, 2007).

Class D OXA β-lactamases that are able to inactivate carbapenems have also been found in strains of the *Acinetobacter baumannii - Acinetobacter calcoaceticus* complex. These include OXA-23, OXA-40 and OXA-58 (Donald *et al*, 2000; Bou *et al*, 2000a; Poirel *et al*, 2005), whilst OXA-51is found only in *Acinetobacter baumannii* (Brown *et al*, 2005; Turton *et al*, 2006).

Outer membrane protein (OMP) modifications

It has been observed in a number of *Acinetobacter baumannii* outbreaks that there is a reduction in the number of OMPs present on the surface of the organism, which limits the access of antibiotics into the organism (Bou *et al*, 2000; Quale *et al* 2003). It is unknown how much this mechanism affects the resistance of the organism, as it is very hard to measure. A couple of OMPs have been to shown to be involved with resistance to certain drugs, such as the 43-kDa homologue of OprD associated with imipenem resistance or the 29-kDa CarO, which confers resistance to imipenem and meropenem (Limansky *et* al, 2002; Dupont *et al*, 2005)

Efflux Pumps

The efflux pumps are specialised molecular pumps that remove toxic compounds from the interior of bacterial cells. There are a variety of families of efflux pumps. The most common families are; the major facilitator family; the small multidrug resistance superfamily; the multidrug and toxic extrusion superfamily and the resistance-nodulation-cell division family (Poole, 2005). In *Acinetobacter baumannii - Acinetobacter calcoaceticus* complex, a member of the resistance-nodulation-cell division family, called the AdeABC efflux pump, has been well characterised. It removes aminoglycosides, cefotaxime, tetracyclines, erythromycin, chloramphenicol, trimethoprim and fluoroquinolones (Perez *et al*, 2007). Over-expression of the AdeABC efflux pump has been theorised to provide high level resistance to carbapenems (Marque *et al*, 2005).

Aminoglycoside resistance

As well as the efflux pump mentioned above, *Acinetobacter baumannii - Acinetobacter calcoaceticus* complex also produces aminoglycoside modifying enzymes (AMEs). There are a variety of these including acetyltransferases, nucleotidyltransferases and phosphototransferases. These AMEs are widespread throughout the world and are expressed by a wide variety of *Acinetobacter baumannii* strains (Peleg *et al*, 2008).

Quinolone resistance

Quinolone resistance is primarily mediated by structural changes in DNA gyrase, caused by mutations in the *gyrA* and *parC* genes (Seward & Towner, 1998). These modifications mean that the quinolone has a lower affinity for the enzyme-DNA complex and as a result the quinolone cannot halt the DNA gyrase effectively. The efflux pump also acts a resistance mechanism for quinolones (Perez *et al*, 2007).

Tetracycline resistance

There are two separate mechanisms of resistance to tetracyclines described in *Acinetobacter baumannii - Acinetobacter calcoaceticus* complex. The first are specific transposon-mediated efflux pumps, known as TetA and TetB. TetA drives the removal of tetracycline, whilst TetB removes tetracycline and minocycline (Perez *et al*, 2007; Peleg *et al*, 2008). The second mechanism is a ribosomal protection protein, which shields the ribosome from the action of tetracycline (Ribera *et al*, 2003).

Polymyxin resistance

Polymyxin B and E are peptide antibiotics originally isolated in 1947 and are currently used as a last resort for cases of multidrug-resistant *Acinetobacter baumannii*. There have been recent reports of resistance developing to even these antimicrobials throughout the world (Urban *et al*, 2001; Gales *et al*, 2006). Currently the resistance mechanism remains unknown. However it has been shown that small changes to the LPS binding site have led to resistance in *E. coli*, *Salmonella* spp. and *Pseudomonas aeruginosa* (Conrad & Galanos 1989; Peterson *et al*, 1987).

1.4.4 Control of the organism

Pan-resistant strains have been isolated from a variety of hospitals world wide, which means that normal therapies and control strategies are no longer effective. Strategies such as proper observance of hand hygiene and also proper management of antibiotic programmes to slow the development of resistance need to be implemented fully (Giamarellou 2006). Also it is important to try to maintain the strength of currently available antibiotics. This can be achieved by the use of antimicrobials that have more than one target of mechanism of action. Furthermore, β-lactams and fluoroquinolones should not be used together. It is also possible to maintain the effectiveness of antibiotics by making sure they are prescribed in shorter doses and that concentrations are at the correct levels (Peterson, 2005). There also needs to be much greater interaction between the labs and clinicians, so that when a susceptibility of the organism is identified, the clinician should immediately change the antibiotic used (McGowan, 2006).

1.5 Aims of the Project

1 The preparation and characterisation of LPS extracted from *Acinetobacter genospecies 13 TU* and *Haemophilus influenzae* (*Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* LPS was extracted as controls of common gram negative lung pathogens).

2 To ascertain the levels of antibody to *Acinetobacter genospecies 13 TU* and *Haemophilus influenzae* LPS in the normal population, by ELISAs on serum from blood donors. 475 donors were tested to determine the range of levels and see if there are likely to be common epitopes in LPS of each species. This will be done initially by seeing whether people that are high responders to one LPS will also have high levels of response to other levels of LPS and vice versa.

3 Check through use of Inhibition ELISAs for cross-reactivity between the different LPS antigens. Thus seeing if there is a putative target for a passive vaccine.

4 The levels of the innate immune response to the different LPSs will be investigated by measuring the induction of inflammatory cytokines from THP-1 cells using QPCR. This will demonstrate how potent the *Acinetobacter* and *Haemophilus* LPS are and also give us an idea of the base level response in the normal population.

2. Materials and Methods

2.1 Growth and LPS extraction methods

2.1.1 Strains used

Samples were received from the diagnostic laboratories from the Royal infirmary of Edinburgh (RIE), as follows

4 samples of *Haemophilus influenzae*.

2 samples of *Pseudomonas aeruginosa*.

2 samples of *Klebsiella pneumoniae*.

2 samples of *Escherichia coli*.

8 samples from *Acinetobacter* genospecies 13 TU.

Table 2.1: strains received

Species	Strain	Source
Haemophilus influenzae	MPRL 4790	Respiratory Tract
Haemophilus influenzae	MPRL 4838	Respiratory Tract
Haemophilus influenzae	MPRL 4839	Respiratory Tract
Haemophilus influenzae	MPRL 4840	Respiratory Tract
Pseudomonas aeruginosa	MPRL 4789	Respiratory Tract
Pseudomonas aeruginosa	MPRL 4845	Respiratory Tract
Klebsiella pneumoniae	MPRL 4841	Respiratory Tract
Klebsiella pneumoniae	MPRL 4844	Respiratory Tract
Escherichia coli	MPRL 4842	Respiratory Tract
Escherichia coli	MPRL 4843	Respiratory Tract
Acinetobacter genospecies 13 TU	MPRL 4793	Blood
Acinetobacter genospecies 13 TU	MPRL 4799	Blood
Acinetobacter genospecies 13 TU	MPRL 4800	Blood
Acinetobacter genospecies 13 TU	MPRL 4801	Blood
Acinetobacter genospecies 13 TU	MPRL 4802	Respiratory Tract
Acinetobacter genospecies 13 TU	MPRL 4803	Blood
Acinetobacter genospecies 13 TU	MPRL 4808	Respiratory Tract
Acinetobacter genospecies 13 TU	MPRL 4809	Respiratory Tract

The strains received and where they were isolated from in the patient.

2.1.2 Growing the Strains

The *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli,* and *Acinetobacter* genospecies 13 TU strains were grown in 100 ml of nutrient broth overnight at 37°C in an orbital incubator. The culture was gram stained to make sure that it was a pure growth of gram negative rods, the broth was also streaked out onto a nutrient agar plate, except for *Acinetobacter* genospecies 13 TU which was streaked onto blood agar. These were then incubated over night at 37°C to make sure that there was only one colony type. A 10 ml volume of the initial growth broth was added to each of 6 2L conical flasks containing 1L of nutrient broth. These were incubated overnight at 37°C in an orbital incubator. Again the cultures were gram stained and plated out as above to test for purity.

Haemophilus influenzae was grown differently due to the additional growth requirements that it requires. It was grown as above except that the growth medium was brain heart infusion (BHI) with 5% fildes extract (Oxoid, Basingstoke, UK) as suggested by Bergeron *et al* (1987), with bacitracin to remove any Gram-positive contamination. The purity of the organism was tested by plating it on chocolate blood agar. Otherwise it was grown in the same manner as the other organisms.

2.1.3 Harvesting the bacteria

The broth containing the bacterial growth was centrifuged in a Sorval RC 26 at an rcf of 16911g for 15 minutes at 4ºC using an SLA 3000 rotor. This was repeated until all 6L of bacteria had been pelleted. The pellets were re-suspended in phosphate buffered saline (PBS, 50nM, pH 7.4) and pooled together. This was then spun down in a Sorval RC 26 at an rcf of 15146g for 15 minutes at 4ºC using an SLA 1500. This was

repeated twice more, after which the pellets were frozen at -20ºC. They were then freeze dried overnight using a freeze drier.

2.1.4 Extracting the LPS

The LPS was extracted using the aqueous phenol method, as developed by Westphal & Jann (1965) and described by Hancock & Poxton (1988). The freeze-dried bacteria were weighed and were re-suspended in pyrogen-free water at a concentration of 5% w/v. The bacterial suspension was heated in a boiling bath to 65ºC, as was an equal volume of 90% (w/w) aqueous phenol. The two solutions were mixed together and kept at 65ºC for 15 minutes stirring every two to three minutes. The mixture was then placed on ice and allowed to cool to allow the layers to separate. This was then centrifuged in the Sorval RC 26 centrifuge at an rcf of 15146g for 15 minutes at 4ºC in a SLA 1500 rotor. The top layer of the phenol was removed and placed in a new tube and re-centrifuged in the Sorval RC 26 at an rcf of 14476g for 15 minutes at 4ºC in a SA – 600 rotor. Again the top layer was removed and placed into pre-prepared dialysis membrane (Viking 32/32) and left in a container with a running tap overnight to remove the phenol from the solution. The sample was centrifuged again in the Sorval Rc 26 at an rcf of 14476g to remove any impurities that may have remained. Now the sample was centrifuged in a Sorval ultracentrifuge at an rcf of 100 000g for 3 hours at 4ºC. It was removed and the supernatant was discarded. The pellet was re-suspended with pyrogen free water using a syringe fitted with a 26 guage needle and then re-centrifuged in the ultra centrifuge as above. The pellet was once again re-suspended in about 4ml of pyrogen free water and transferred into a pre-weighed plastic bijou and frozen overnight at -20ºC. It was then freeze-dried overnight and weighed to determine the weight of LPS obtained from the extraction.

2.1.5 Repurification of LPS.

The Vogel's method for the repurification of LPS after initial extraction, developed by Hirshfield *et al* (2000), was used to remove any remaining cell surface protein from the LPS. The freeze dried LPS was suspended in 0.2% triethylamine at a concentration of 5mg/ml. A 500µl volume of the suspension was transferred into a 1.5ml microcentrifuge tube and 100µl of 3% sodium deoxycholate solution (creating a 0.5% concentration of sodium deoxycholate). This was mixed briefly and then 600µl of water-saturated phenol was added. This new solution was vortexed intermittently for 5 minutes, the phase was then allowed to separate for 5 minutes at room temperature. The sample was then placed on ice for 5 minutes and then centrifuged at 10000g for 2mins.

The top aqueous layer (including any white material at the phase interface) was transferred to another 1.5ml microcentrifuge tube. The lower phenol layer was re-extracted with 600µl of 0.2% triethylamine / 0.5% sodium deoxycholate, as described above. The top aqueous layer was pooled with the previously collected top aqueous layer.

The pooled samples were divided into two 1.5ml microcentrifuge tubes and both extracted with 600µl of water-saturated phenol. The top aqueous layers were transferred to another 1.5ml microcentrifuge and the volume was measured. 1 volume of 300mM sodium acetate was added to 9 volumes of the aqueous phase and mixed briefly.

Volumes of 400µl of the above samples were transferred to separate microcentrifuge tubes and 1.2ml ethanol was added (making a concentration of 75% ethanol in each tube). This was allowed to precipitate at -20ºC for 1 hour. The samples were then

centrifuged at 10000g for 10 minutes. The supernatant was discarded and the precipitate was resuspended and pooled in cold ethanol (-20ºC). This was wash precipitated by centrifugation at 10000g for 10 minutes. The supernatant was discarded and the precipitate allowed to dry at room temperature. The dried precipitate was dissolved, at a concentration of 5mg/ml, in 0.2% triethylamine and stored at -20ºC.

2.2 Staining and gel methods

2.2.1 Polyacrylamide gel electrophoresis (PAGE)

A 10µl volume of the LPS dissolved in the 0.2% triethylamine was added to an equal volume of double strength sample buffer in a microcentrifuge tube. This was then heated to 100ºC for three minutes. A 15µl volume of the sample and buffer solution was added into a well in the NuPAGE 10% acrylamide mini-gel (Invitrogen). The gel was then run at 200 volts in the XCell *Sure*Lock^{tm} Mini-Cell (Invitrogen) for 30-40 minutes. The gel was then carefully removed from the casing and either placed in LPS fixative overnight for a silver stain the next day or used directly in a Western blotting experiment

2.2.2 Western Blotting

A rectangle of nitrocellulose membrane 2cm longer and 1 cm wider than the gel area was cut out marked on one side with a soft pencil mark. The nitrocellulose membrane was placed into Western blot electrode buffer and left for ten minutes. Four sheets of blotting paper larger than the nitrocellulose membrane were cut out and soaked in Western blot electrode buffer for 10 minutes. The gel was placed in the Western

electrode buffer for 10 minutes to remove salts. Two sheets of blotting paper were placed in the cathode side of the Western blot gel cassette and then the gel was placed on the blotting paper. The nitrocellulose membrane was carefully placed on the gel, making sure no air bubbles were trapped and the remaining two pieces of blotting paper were laid on top of the nitrocellulose membrane. The cassette was then sealed and placed into the Flowgen B4-0200 electro-blotting unit. The cassette was then covered in electrode buffer and the blotting tank was run at a constant voltage of about 5V, which gave a constant current of 40mA. The Western blot was run over night at a temperature of 4ºC. At the end of the transfer, the cassette was removed from the tank and the nitro cellulose membrane was transferred into a box and covered in 50ml of Tris buffered saline (TBS).

2.2.3 Silver Stain for LPS

The PAGE gel was placed in plastic box and covered in LPS fixative (25% propan-2-ol, 7% acetic acid) overnight to fix the LPS into the gel. After this step, all other solutions were agitated on a rocker platform at a rate of 10-20 tilts per minute.
The fixative was discarded and the gel was oxidised in 154ml of oxidiser solution (0.7% periodic acid in dilute fixative) for 15 minutes. The oxidiser was discarded and the gel was washed four times in 200ml of pyrogen-free water for one hour. The water was discarded between each wash. Ammoniacal silver nitrate solution (100ml 0.076% NaOH, 0.014% ammonia solution, 0.078% silver nitrate solution in pyrogen-free water) was freshly prepared and then added to the gel and left for 15 minutes and then discarded down a fume cupboard sink with lots of water. The gel was washed four times in 200ml of pyrogen-free water for 10 minutes. Developer (200ml 0.005% citric acid in .019% formaldehyde solution) was freshly prepared and poured onto the gel

until the correct staining intensity was achieved. The gel was then washed twice more for 5-10 minutes each time in 200ml of pyrogen free water. The gel was finally scanned and saved.

2.2.4 Colloidal Gold Stain

The nitrocellulose membrane from the Western blot was placed into 100ml of Tween-20 Tris-Buffered Saline (TTBS) and washed for 20 minutes and repeated twice more. The nitrocellulose membrane was then rinsed with 100ml of pyrogen-free water for 2 minutes, this was also repeated twice. Colloidal Gold Total Protein Stain (50ml: Biorad) was poured onto the nitrocellulose membrane; this was left for 1-2 hours until all the bands had appeared. Once the bands had appeared, the Colloidal Gold Stain was poured off and the membrane was washed with 100 ml of pyrogen free water for 1 minute; this was repeated twice and the membrane was scanned and saved.

2.2.5 Limulus Amoebocyte Lysate (LAL) assay

The endotoxic activity of the different LPS extractions was ascertained through the use of an endpoint LAL assay. This was done using a Pyrochrome LAL kit (Associates of Cape Cod), which is based on the work done by Levin and Bang (1964). The kit was used following the instructions of the manufacturers. The Pyrochrome was re-suspended in 3.2 ml of Pyrochrome buffer and kept on ice. For the creation of a standard curve, firstly 4.0 ml of LAL reagent water (LRW) was added to 0.2 ng of *E. coli* endotoxin to create a concentration of 0.5 endotoxic units (EU)/ml. This was then used at a doubling dilution from 0.5 EU/ml with seven doubling dilutions, to make a seven point standard curve. All the LPS samples were diluted to 1 ng/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml and 0.1 pg/ml in LRW. These

dilutions were then added at 50 µl per well of a 96 well plate. Pyrogen free water was used as a control. The Pyrochrome reagent (50 µl) was added to all samples and the control. The plate was then shaken gently and incubated at 37ºC for 30 minutes. Acetic acid (50%, 25 µl) in Pyrogen free water was added to each well to stop the reaction. The plate was then read at 405nm on an Anthos 2001 automated plate reader.

2.3 Antibody work

2.3.1 Serum samples

After ethical approval was granted, the serum samples were obtained from 500 healthy blood donors from the Southeast Scotland Blood Transfusion Service. These were collected and stored at -20ºC. The serum samples were diluted 1 in 200 in serum and conjugate dilulent (0.05M sodium phosphate buffer pH 7.4, 0.85% w/v sodium chloride, 0.05% Tween-20, 0.02% w/v sodium azide) and also stored at -20ºC for four months before more was made.

2.3.2 Pooled serum

Pooled serum was made by mixing together 100µl of serum from 250 blood donors to make 25ml. This was stored at -20ºC and was diluted 1 in 50 in serum and conjugate dilulent before use.

2.3.3 LPS and Polymyxin B complex formation.

The repurified LPS was diluted to a concentration of 1mg/ml (w/v) and 1 ml was mixed with an equal volume of polymyxin B also at a concentration of 1mg/ml (w/v). The solution was sonicated at 10μm for 30 seconds. The solution was then gently rotated for 1.5 hours at room temperature after which it was re-sonicated as above. The solution was dialysed overnight (Spectra pore membrane MWCO 2000) against pyrogen free water to remove any non complexed LPS or polymyxin B. The complexed LPS and polymyxin B was stored at -20ºC.

2.3.4 Enzyme Linked Immunosorbant Assay (ELISA) protocol.

The procedure was taken from Allan *et al*. (1995).

The LPS and polymyxin B complex was diluted 1 in 50 in antigen diluent (0.05M carbonate/bicarbonate, pH 9.6, containing 0.02% sodium azide) and 100μl was pipetted into each well in a 96 well Medisorp plate (Nunc) and incubated at room temperature overnight. The plates were washed three times in ELISA wash buffer (PBS, pH 7.4 containing Tween-20, 0.05% w/v) and then blocked with 100μl of 3% fish gelatine (Sigma) in PBS with 0.02% sodium azide and incubated at room temperature overnight. The plates were washed three times with ELISA wash buffer and twice with pyrogen-free water. They were then stored at -20ºC until required.

A volume of 100μl of the 1 in 200 diluted serum samples collected from the Blood Transfusion Service were placed into the wells and incubated at 37ºC for 1.5 hours and then washed three times in ELISA wash buffer. Anti-human IgG alkaline phosphatase conjugate goat antibody (Sigma) was diluted 1 in 1000 in serum and conjugate dilulent and 100μl per well was pipetted onto the plate. The plate was incubated for 1.5 hours at 37ºC and then washed three times in ELISA wash buffer.

Alkaline phosphatase substrate was prepared at a concentration of 1mg/ml (w/v) in 0.05M carbonate bicarbonate buffer pH 9.8 containing 1mM $MgCl_2$. This was added to the plate and incubated for 30-40 minutes at room temperature. The plates were then read in an Anthos 2001 plate reader at an optical density of 405nm. The 96^{th} well of each plate was a control serum sample from a known healthy donor.

2.3.5 ELISA inhibition assay

This protocol was developed from Allan *et al* (1995).

The LPS was diluted 1 in 8 in antigen diluent (0.05M carbonate/bicarbonate, pH 9.6, containing 0.02% sodium azide) and 100µl per well was pipetted into a 96 well Medisorp plate (Nunc) and incubated at room temperature overnight. The plates were washed 3 times with ELISA wash buffer (PBS, pH 7.4 containing tween 20, 0.05% w/v) and then the plates were blocked with 200µl of 3% fish gelatine (Sigma) in tris buffered saline with 0.02% sodium azide. The plates were incubated overnight at room temperature and then washed three times in ELISA wash buffer and twice with pyrogen free water. LPS extracted from the strains of bacteria was mixed at concentrations of; 1mg/ml, 200mg/ml, 40mg/ml, 8mg/ml, 1.6mg/ml, 0.32mg/ml, 0.06mg/ml and 0.01mg/ml with the 1 in 50 dilution of the pooled serum samples. They were incubated for 30 minutes at 37ºC. The dilutions of LPS (100 µl) and pooled serum were plated onto the plate and incubated for 1.5 hours at 37ºC. The plate was then washed three times in ELISA wash buffer. Anti-human IgG alkaline phosphatase conjugate goat antibody (Sigma) was diluted 1 in 20000 in serum and conjugate dilulent and 100µl per well was pipetted onto the plate. The plate was incubated for 1.5 hours at 37ºC and then washed three times in ELISA wash buffer. Alkaline phosphatase substrate was prepared at a concentration of 1mg/ml (w/v) in

0.05M carbonate bicarbonate buffer pH 9.8 containing 1mM $MgCl_2$. This was added to the plate and incubated for 30-40 minutes at room temperature. The plates were then read in an Anthos 2001 plate reader at an optical density of 405nm. Dilutions of control LPS from *E. coli* O18 were used as a positive control.

2.4 Cell Culture

2.4.1 Cell line

The cell line used for this study was the human acute monocytic leukaemia cell line (THP-1). This cell line was derived from a 1 year old human male, (Tsuchiya *et al*, 1980).

2.4.2 Media used

The cells were grown initially in a start up culture of RPMI-1640 medium (Sigma), 20% foetal bovine serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma), 6mM L- glutamine (Sigma) and 1% pen-strep (Sigma) (RPMI/20%FBS/HEPES/L-Glu/PS+G). For routine growth once the cells were established a medium containing RPMI-1640 with 10% FBS, 6mM L-glutamine and 1% pen-strep (RPMI/10%FBS/L-Glu/PS+G) was used. To differentiate the cells a solution of 20μM vitamin D3 (sigma) was added to the medium to make a final concentration of 20nM. This was added every day for three days. The cells were incubated at 37ºC with 5% CO_2 in a CO_2 incubator. The cells were placed into liquid nitrogen storage in a freezing medium containing 30% sterile glycerol (Sigma) and 70% FBS.

2.4.3 Cell preparation for cytokine production

The cells were counted using an improved Neubauer counter and re-suspended at a concentration of $1x10^6$ cells/ml. 1ml of the cells was plated in 14 wells of a 24 well cell culture plate (Costar). This was incubated at 37ºC in a CO_2 incubator for 1 hour to allow the cells to re-attach to the plastic. The cells were then stimulated with LPS from the following species and strains.

Table 2.2: Strains used for TNF-α production.

Haemophilus influenzae	MPRL 4839
Haemophilus influenzae	MPRL 4840
Pseudomonas aeruginosa	MPRL 4789
Pseudomonas aeruginosa	MPRL 4845
Klebsiella pneumoniae	MPRL 4841
Klebsiella pneumoniae	MPRL 4844
Escherichia coli	MPRL 4842
Escherichia coli	MPRL 4843
Acinetobacter genospecies 13 TU	MPRL 4802
Acinetobacter genospecies 13 TU	MPRL 4809

The LPS was added at 100ng/ml to the first well and then underwent ten-fold dilutions in the other wells until it reached a concentration of 0.1pg/ml. This was incubated for 4 hours at 37ºC in a CO2 incubator. Thereafter the cells underwent RNA extraction.

2.5 RNA analysis

2.5.1 Primers

The Primers were designed using the primer 3 website (http://fokker.wi.mit.edu/primer3/input.htm) and ordered from VhBio. The genes of interest were; 18S Ribosomal RNA, Tumour Necrosis Factor α (TNF-α), Interferon-γ (IFN-γ), Interleukin-1β (IL-1β), Interleukin-8 (IL-8) and Interleukin-10 (IL-10).

Table 2.3: Primers of the genes of interest.

Gene of Interest	Primers	Size
18S Ribosomal RNA Forward	CTCAACACGGGAAACCTCAC	151 bp
18S Ribosomal RNA Reverse	ATGCCAGAGTCTCGTTCGTT	
TNF-α Forward	AGCTGTTGAATGCCTGGAAG	151 bp
TNF-α Reverse	TGTTGGGGAGAAGGAGAATG	
IFN-γ Forward	CGGGGAGTACTGAGAAGCAG	155 bp
IFN-γ Reverse	CAGTTCCTTGGTGGCTGAGT	
Il-1β Forward	TGCACTGCTGTGTCCCTAAC	151 bp
Il-1β Reverse	TCTTTCAACACGCAGGACAG	
Il-8 Forward	CAGGAATTGAATGGGTTTGC	151 bp
Il-8 Reverse	TGGATCCTGGCTAGCAGACTA	
Il-10 Forward	CCAAGACCTGGGTTGTCATC	150 bp
Il-10 Reverse	CATCATGAGGAGGCCAGATT	

2.5.2 DNA extraction

The DNA was extracted from the THP-1 cells using NucleoSpin® Tissue (Machery-Nagel)

The THP-1 cells were used at a concentration of $2x10^6$ cells/ml. The cells were centrifuged at 1500g for 5 minutes at room temperature and the medium discarded. The cells were re-suspended in a 200µl of buffer T1. 25µl of proteinase K solution and 200µl of buffer B3 was added to the solution. The solution was incubated at 70ºC for 10-15 minutes.

Ethanol (210µl,100%) was added to the sample and vortexed vigorously to adjust the binding conditions of the DNA. Each sample was added to a NucleoSpin® Tissue column, loaded into a collection tube, and centrifuged at 11000g for 1 minute to bind the DNA to the filter. The flow-through was discarded and the column replaced into the collection tube.

Buffer BW (500µl) was added to the column and centrifuged at 11000g for 1 minute. The flow-through was discarded and the column was replaced into the tube. Buffer B5 (600µl) was added to the column and centrifuged at 11000g for 1 minute. The flow-through was discarded and the column replaced into the collection tube. The column was centrifuged at 11000g for 1 minute to dry the membrane and remove any residual ethanol.

The column was then placed in a 1.5ml microcentrifuge tube and 100µl of pre-warmed (70ºC) elution buffer BE was added. This was incubated at room temperature for 1 minute and then centrifuged at 11000g for 1 minute.

The samples were then stored at -20ºC before use.

2.5.3 RNA extraction

The RNA was extracted using the Purelink™ Micro-to-Midi total RNA purification system (Invitrogen). Fresh RNA lysis buffer was prepared by adding 1% (v/v) to the lysis buffer. The lysis buffer was pipetted onto the THP-1 cell monolayer at 0.3ml in each well, once the supernatant had been removed. This was then pipetted up and down until the cells appeared lysed. The lysis buffer was then passed through a 19 gauge needle attached to an RNase free syringe. This was then transferred into an RNase free 1.5ml Eppendorf tube. An equal volume of 70% ethanol was added to the lysis buffer. This mixture was vortexed thoroughly to disperse any precipitate.

Then 700µl was transferred to an RNA Spin Cartridge inserted into a collection tube. The tube was centrifuged at 12000g for 30 seconds at room temperature and the flow-through was discarded. The spin cartridge was replaced in the collection tube. Wash buffer 1 (700µl) was added to the spin cartridge and centrifuged at 12000g for 30 seconds at room temperature. The flow-through and collection tube were discarded and the spin cartridge was replaced with an RNA wash tube. Wash Buffer 2 (500µl) was placed in the spin cartridge and centrifuged at 12000g for 30 seconds at room temperature. The flow-through was discarded and the spin cartridge was replaced in the tube. This step was repeated once.

The spin cartridge was centrifuged at 12000g for 1 minute at room temperature to dry the membrane. The collection tube was discarded and the spin cartridge was placed in an RNA recovery tube. RNase free water (50ul) was added to the spin cartridge and left for 1 minute at room temperature to incubate. This was then centrifuged at 12000g for 2 minutes at room temperature to elute the RNA. The eluted RNA was stored at -70ºC.

2.5.4 Reverse transcription of the RNA

This was carried out using the Ambion RETROscript Reverse Transcription for RT-PCR. Total RNA (1-2µg) and 2µl Oligo(dT) were mixed in a 1.5 ml Eppendorf tube and made up to 12µl with nuclease-free water. This mixture was briefly mixed and heated for 3 minutes at 70-85ºC. The tubes were then removed to ice before being centrifuged briefly and returned to ice. 10X RT Buffer (2µl), 4 µl dNTP mix, 1 µl RNase inhibitor and 1 µl reverse transcriptase were added to give a final volume of 20 µl. The mixture was mixed gently and centrifuged briefly before being incubated for 1 hour at 42-44ºC. The tubes were then incubated at 92ºC for 10 minutes to inactivate the reverse transcriptase. The RNA had now been converted into complementary (c)DNA. The samples were stored at -20ºC for future use.

2.5.5 Polymerase Chain Reaction (PCR)

In a 200µl PCR tube 5µl of PCR buffer (VhBio), 2µl of DnTPs (Amersham Pharmacia Biotech), 1µl of PCR Enhancer (VhBio), 1.5µl Forwards Primer, 1.5µl Reverse Primer, 4µl DNA and 0.5µl Moltaq (VhBio) were mixed. Nuclease free water 34.5 µl was added to make the final volume 50µl. This was all carried out on ice.
The tubes were placed in a TC 412 Techne PCR machine and heated to 95ºC for 5 minutes for initial denaturisation. The samples were then denatured at 95ºC for 30 seconds, then annealed at 56ºC for 30 seconds and then extended at 72ºC for 30 seconds. This was repeated for another 39 cycles. Then there was a final extension at 72ºC for 10 minutes.
The samples were mixed with loading buffer at a concentration of 90%(v/v) then run on a 2% agarose gel for 3 hours with a 100bp ladder (promega). The gel was imaged using a Gel Imager.

2.5.6 Quantitative PCR (QPCR)

2.5.6.1 Primer concentration testing

Firstly, it was necessary to calculate the ideal concentration of primers to be used for each gene. To this end, four dilutions of the primers were prepared. These dilutions were 50nM, 100nM, 200nM and 400nM and they were tested with DNA extracted from THP-1 cells. The 50nM concentration contained 1µl of forward primer (from the 1µM stock solution), 1µl of reverse primer (the 1µM stock solution), 2µl of DNA, 6µl of nuclease free water and 10µl of SYBR green (Sigma-Aldrich). The 100nM concentration contained 2µl of forward primer (from the 1µM stock solution), 2µl of reverse primer (the 1µM stock solution), 2µl of DNA, 4µl of nuclease free water and 10µl of SYBR green. The 200nM concentration contained 4µl of forward primer (from the 1µM stock solution), 4µl of reverse primer (the 1µM stock solution), 2µl of DNA and 10µl of SYBR green. The 400nM concentration contained 0.8µl of forward primer (from the 10µM stock solution), 0.8µl of reverse primer (the 10µM stock solution), 2µl of DNA, 6.4µl of nuclease free water and 10µl of SYBR green. A negative control was used for all primers. This was the same as for the 100nM primer concentration, but without any DNA. The make up of the negative control was thus as follows: 2µl of forward primer (from the 1µM stock solution), 2µl of reverse primer (the 1µM stock solution), 6µl of nuclease free water and 10µl of SYBR green. Each primer dilution and negative control was carried out in triplicate.

The plate was then placed into a Stratagene Mx3000P QPCR machine and heated to 95ºC for 5 minutes for initial denaturisation. The samples were then denatured at 95ºC for 20 seconds, annealed at 56ºC for 20 seconds and then extended at 72ºC for 20 seconds. This was repeated for another 39 cycles. There was a final cycle of heating

to 95ºC for 1 minute, annealed at 50ºC for 30 seconds and then a final heating at 95ºC for 30 seconds.

2.5.6.2 Dilution of antigen testing for complimentary DNA (cDNA).

After the best concentrations of primer were calculated, it was also necessary to work out which two concentrations of the cDNA to use. The THP-1 cells had been challenged with 7 different concentrations of LPS, ranging from 100ng/ml to 0.1pg/ml. It was decided that only two of these concentrations for each gene would need to be tested. To test which of the cDNAs would be best to use, all the different cDNAs were used in a QPCR experiment. All 7 concentrations of LPS used to challenge the THP-1 cells were tested, using the cDNA from the THP-1 cells challenged with *Acinetobacter genospecies 13 TU* 4809 LPS.

This was carried out as above with all the primers being used at the 200nM concentration, except for TNF-α, which used the 400nM primer concentration. So the make up of the solution for all the genes, except TNF-α, in each tube was 4µl of forward primer (from the 1µM stock solution), 4µl of reverse primer (the 1µM stock solution), 2µl of cDNA (from the cells challenged with LPS from A.b. 4809), 0µl of nuclease free water and 10µl of SYBR green. The solution for TNF-α was as follows: 0.8µl of forward primer (from the 10µM stock solution), 0.8µl of reverse primer (the 10µM stock solution), 2µl of cDNA, 6.4µl of nuclease free water and 10µl of SYBR green.

The positive control was the 18S ribosomal RNA primers at a concentration 200nM, using the DNA extracted from the THP-1 cells directly. The negative controls were the same as in experiment 2.5.6.1. All the samples were done in triplicate. The plate was run in the Stratagene Mx3000P, as in experiment 2.5.6.1.

2.5.6.3 QPCR of the cDNAs against all the genes.

The final stage measured the cDNA, at the concentrations that were decided to be the most effective in experiment 2.5.6.2, against all the genes of interest. This was carried out as in the previous experiments. The genes of interest were used at the primer concentration of 200nM except TNF-α, which was used at the primer concentration of 400nM. The cDNA used was from the cells challenged by the LPS dilutions of 10ng/ml and 1ng/ml for 18S ribosomal RNA, TNF-α, IL-1β and IL-10. The cDNA used for IFN-γ and IL-8 was from the cells challenged by the LPS dilutions of 100ng/ml and 10ng/ml. The positive control and the negative controls were the same as before. All the samples were run in triplicate.

The samples were run under the same heating conditions as in the previous experiments.

2.6 Statistical analysis

All statistical analysis for the ELISA data was carried out using Microsoft Office Excel and Minitab 15 statistical software. All the QPCR data were analysed using MxPro QPCR software version 3.0 and Minitab 15 statistical software. All the data sets from the ELISA results were tested against one another using 2 sample t-tests. This same technique was also used for the QPCR data.

3. Results

3.1 Extraction and purification of Lipopolysaccharide

3.1.1 Amount of LPS extracted from the bacterial strains

The LPS was extracted from between 0.87g and 2.5g of lyophilised bacteria. The extraction was carried out using the aqueous phenol method developed by Westphal & Jann (1965), modified by Hancock & Poxton (1981), as described in chapter 2.1.4.

The bijou bottles were weighed, before the re-suspended LPS in pf water was added. They were then weighed again, after the LPS had been lyophilised, and the extra mass was taken to be the mass of the LPS extracted from the bacteria. These results are detailed in table 3.1.

Table 3.1: Mass of LPS extracted from the bacterial strains.

Bacterial Strain	Weight of dried bacteria	Weight of extracted LPS	Percentage of total bacterial weight
A. g 13 TU 4793	1.00g	0.025g	2.50%
A. g 13 TU 4799	1.36g	0.026g	1.91%
A. g 13 TU 4800	2.40g	0.05g	2.08%
A. g 13 TU 4801	1.20g	0.016g	1.33%
A. g 13 TU 4802	2.00g	0.037g	1.85%
A. g 13 TU 4803	1.32g	0.041g	3.10%
A. g 13 TU 4808	2.10g	0.045g	2.14%
A. g 13 TU 4809	2.00g	0.096g	4.80%
H.i. 4790	0.87g	0.018g	2.06%
H.i. 4838	2.00g	0.049g	2.45%
H.i. 4839	1.92g	0.01g	0.52%
H.i. 4840	1.00g	0.028g	2.80%
E.c. 4841	2.00g	0.042g	2.10%
E.c. 4844	1.60g	0.037g	2.31%
K.p. 4842	1.30g	0.031g	2.38%
K.p. 4843	1.20g	0.028g	2.33%
P.a. 4789	2.50g	0.035g	1.40%
P.a. 4845	1.70g	0.027g	1.59%

As can be seen in table 3.1, the amounts extracted vary between 96 mg, from *Acinetobacter* genospecies 13 TU 4809, and 10mg from *Haemophilus influenzae* 4839 being the largest and smallest amounts extracted respectively. The weights of the majority of the LPS extracted from the organisms were very similar. This is as was expected, as it has been observed that LPS makes up between 1 and 3% of the total weight of dried bacteria.

3.1.2 Silver stain gels of the LPS extractions.

The silver stain was carried out to see the purity of the LPS extractions. Both the original aqueous phenol extractions and the re-extracted LPS samples were used. Figures 3.1, 3.2 and 3.3 show all the bacterial species and strains used throughout this project in both their original and re-extracted forms they all show the purity of the LPS extracted and also what type of LPS had been extracted from the bacteria. If it is a smooth LPS, it will have a pattern of bands. Each band is made up of the Lipid A moiety and the core polysaccharide, with a number of the polysaccharide repeating units that make up the O-polysaccharide chain attached. This is why there are often a large number of bands, as each band is a different number of the repeating units attached to the core i.e. Lipid A + core + 1 repeating unit, Lipid A + core + 2 repeating unit … Lipid A + core + *n* repeating units. Rough LPS is just made up of the Lipid A + core polysaccharide. An example of a smooth LPS is the *E. coli* O18 on the left side of the figure. The bands can easily be seen. K12 is the positive control for the rough LPS and as can be seen in the figures below, there is only the core present without a banding pattern. A semi rough pattern is one with a small number of repeating units an example can be seen in K12 in Figure 3.1 below.

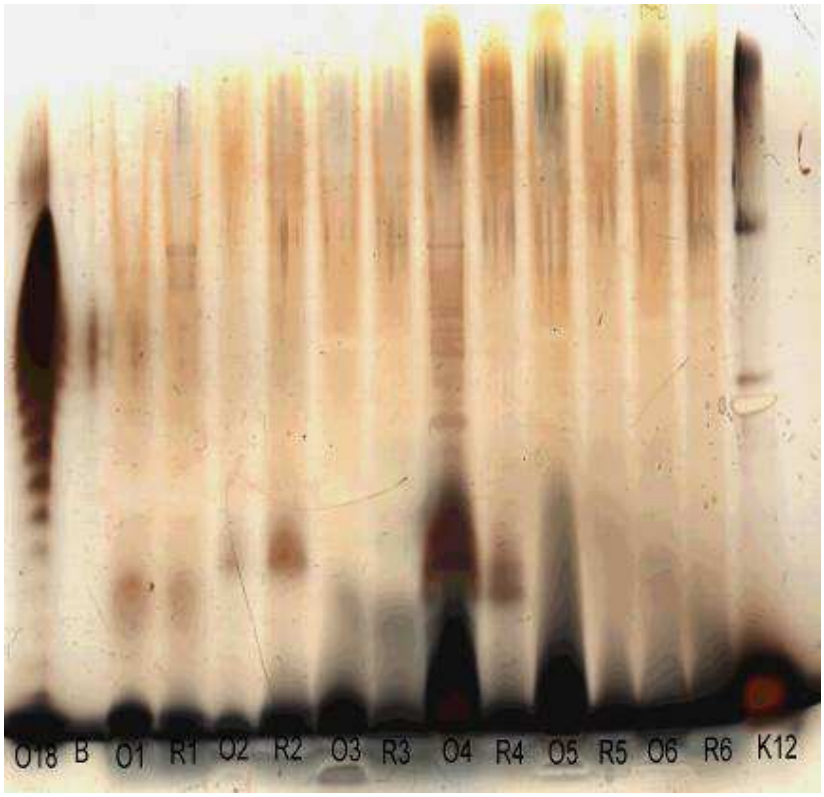


Figure 3.1: Silver stain of *A.* genospecies 13 TU strains A. g 13 TU 4800, A. g 13 TU 4801, A. g 13 TU 4802, A. g 13 TU 4803, A. g 13 TU 4808, A. g 13 TU 4809

O = original extraction, R = re-extracted LPS, B = blank the negative control,
1-A. g 13 TU 4800, 2-A. g 13 TU 4801, 3-A. g 13 TU 4802, 4-A. g 13 TU 4803, 5-A. g 13 TU 4808, 6-A. g 13 TU 4809, O18- *E. coli* positive control for a smooth LPS, K12- *E. coli* positive control for a rough LPS.

The original extractions of *Acinetobacter* genospecies 13 TU 4803 and *Acinetobacter* genospecies 13 TU 4808 both still had some bacterial surface proteins attached, but these had been removed by the re-extraction process, as the re-extracted LPS had no bands remaining. The LPS of most *Acinetobacter* genospecies 13 TU strains appears to be rough on gels. This is due to the make up of the *Acinetobacter* genospecies 13 TU LPS, which does not bind to the silver stain properly. It has been shown by Haseley *et al*, 1998 and Pantophelet *et al*, 2001 that the *Acinetobacter* genospecies 13 TU LPS is normally smooth.

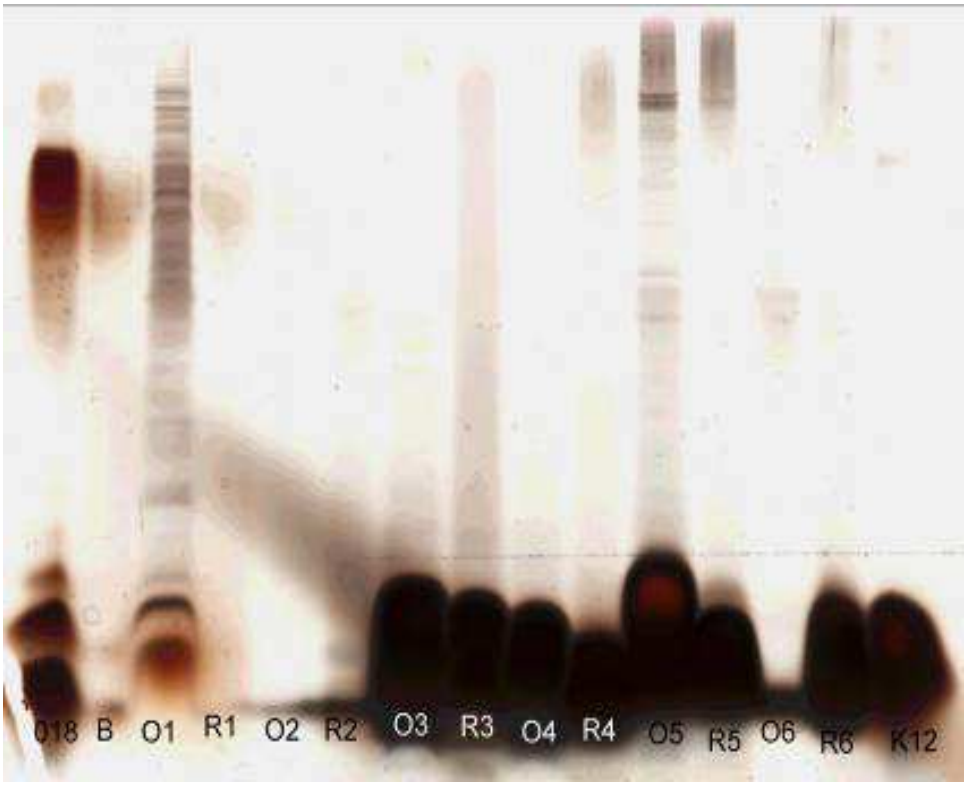


Figure 3.2: Silver stains of strains P.a. 4789, P.a. 4845, H.i. 4790, H.i. 4838, H.i. 4839, H.i. 4840

O = original extraction, R = re-extracted LPS, B = blank the negative control,

1-P.a. 4789, 2-P.a. 4845, 3-H.i. 4790, 4-H.i. 4838, 5-H.i. 4839, 6-H.i. 4839, O18- *E. coli* positive control for a smooth LPS, K12- *E. coli* positive control for a rough LPS.

Figure 3.2 is laid out in the same manner as Figure 3.1, but the gel was run with LPS from different strains. In this case the strains were two *Pseudomonas aeruginosa* strains and four *Haemophilus influenzae* strains. The *Haemophilus influenzae* strains are rough forms and most of them are very pure samples, as there are no banding patterns in either the original or the re-extracted LPS. The original extraction of *Haemophilus influenzae* 4839 still had bacterial proteins attached to the LPS but these were removed by the re-extraction. The original extraction of *Pseudomonas aeruginosa* 4789 also had bacterial proteins attached, but these were removed by the re-extraction. Both *Pseudomonas aeruginosa* 4879 and *Pseudomonas aeruginosa*

4845 seemed to have rough LPS, but *Pseudomonas aeruginosa* like *Acinetobacter* genospecies 13 TU also does not always appear to be smooth when it often is.

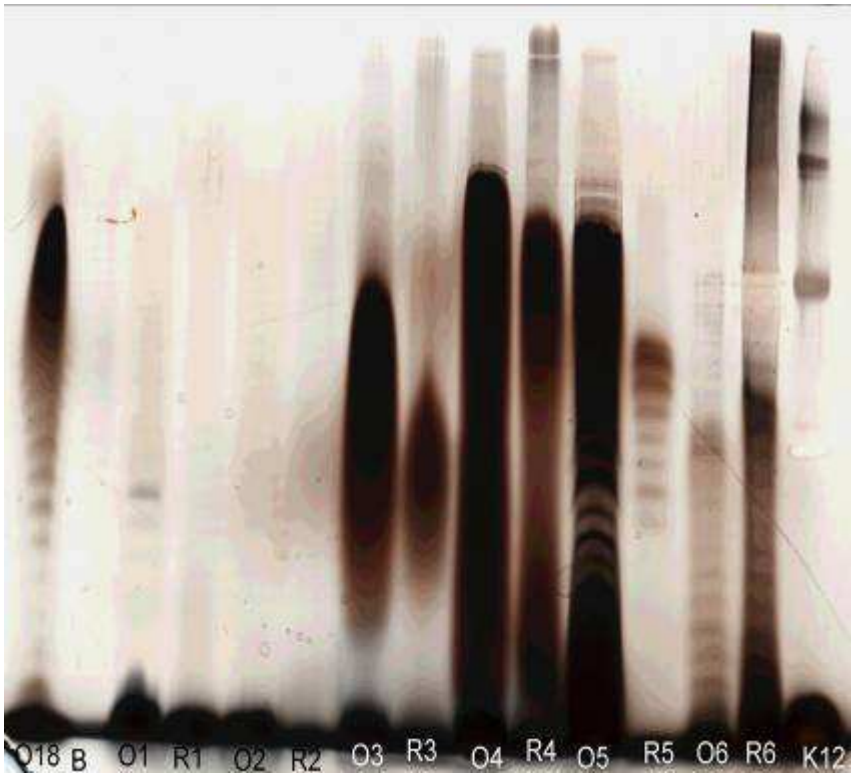


Figure 3.3: Silver stains of strains A. g 13 TU 4793, A. g 13 TU 4799, K.p. 4841, K.p. 4844, E.c. 4842, E.c. 4843

O = original extraction, R = re-extracted LPS, B = blank the negative control,
1-A. g 13 TU 4793, 2-A. g 13 TU 4799, 3-K.p.4841, 4-K.p. 4844, 5-E.c. 4842, 6-E.c. 4843, O18- *E. coli* positive control for a smooth LPS, K12- *E. coli* positive control for a rough LPS.

Figure 3.3 is of a similar layout to the previous two figures, again with LPS from different species. The two *Acinetobacter* genospecies 13 TU species both appear to be rough forms from their appearance on the figure but it is believed, that as in the Figure 3.1 they are in fact smooth. The two *Klebsiella pneumoniae* strains both show the characteristic streak pattern that is common for *Klebsiella pneumoniae* LPS. This streak is due to the small size of the repeating units in *Klebsiella pneumoniae*'s

polysaccharide chain: many and homopolysaccharides. This means that the different bands are very close together, as an increase of one repeating unit does not vary the molecular weight by much. The two *E. coli* strains have a traditional smooth LPS ladder pattern with multiple obvious bands. Both of the *Klebsiella pneumoniae* and *E. coli* strains are similar in both the original extraction and after the re-extractions.

3.1.3 Colloidal Gold total protein stains of the LPS extractions

These experiments were done to measure the total bacterial protein on the LPS extractions. This was to make sure that the LPS was properly purified and thus that the effects we observed, were due to the pure LPS and not due to any bacterial proteins attached to them. The Colloidal gold binds to any proteins present on the nitrocellulose membrane and stains them, so it is easy to see if there are any proteins still attached to the LPS. Figures 3.4, 3.5 and 3.6 below show the purities of the LPS extractions. The negative control is a blank, whilst the positive controls were the mark 12 protein marker (Invitrogen), as it is a definite positive for protein

Figure 3.4: Colloidal Gold stain of *A.* genospecies 13 TU strains A. g 13 TU 4800, A. g 13 TU 4801, A. g 13 TU 4802, A. g 13 TU 4803, A. g 13 TU 4808, A. g 13 TU 4809

O = original extraction, R = re-extracted LPS, B = blank the negative control,
1-A. g 13 TU 4800, 2-A. g 13 TU 4801, 3-A. g 13 TU 4802, 4-A. g 13 TU 4803, 5-A. g 13 TU 4808, 6-A. g 13 TU 4809, P – positive control Mark 12 protein marker (Sigma)

As can be seen in Figure 3.4, the two positive controls both show that protein is present, whereas none of the LPS extractions had any protein appear on the figure and so it is possible to assume that there is no detectable protein in the original and re-extracted LPS.

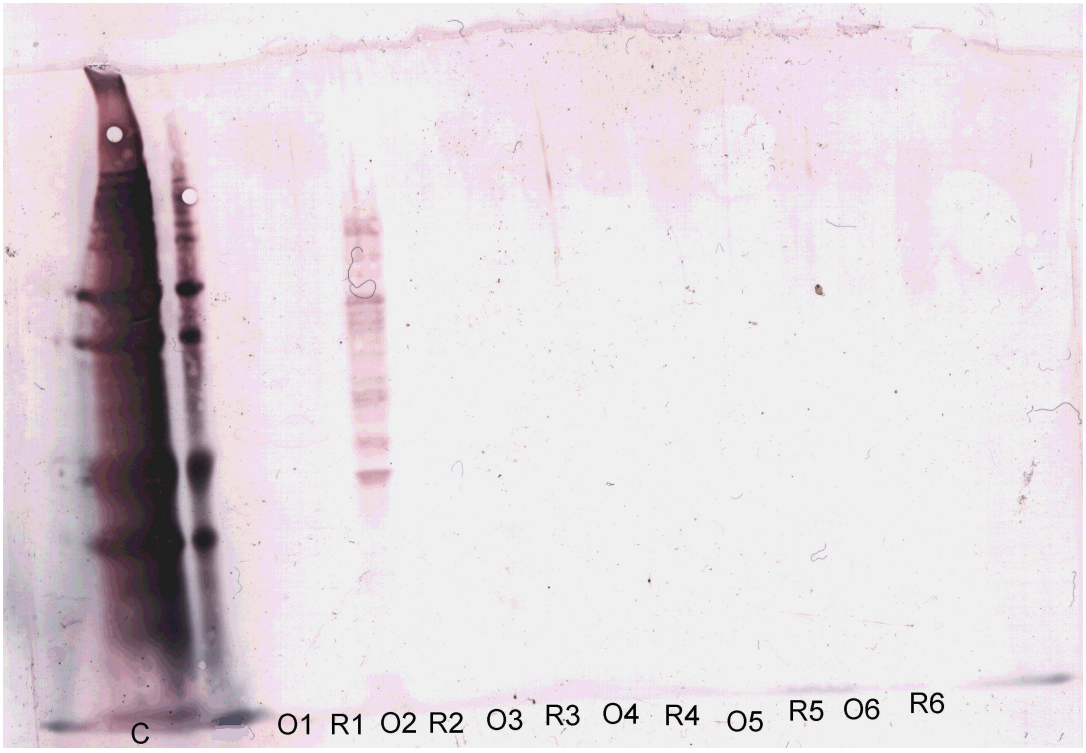


Figure 3.5: Colloidal Gold stain of strains P.a. 4789, P.a. 4845, H.i. 4790, H.i. 4838, H.i. 4839, H.i. 4840

Legend: O = original extraction, R = re-extracted LPS, 1-P.a. 4789, 2-P.a. 4845, 3-H.i. 4790, 4-H.i. 4838, 5-H.i. 4839, 6-H.i. 4839, C – positive control Mark 12 protein marker (Sigma)

In Figure 3.5 there is protein contamination in the original LPS extraction of *P. aeruginosa* 4845 (O2), but there is none in the re-extracted LPS (R2), so the re-extraction process has cleaned this LPS up. The other LPSs have no protein contamination. The positive control is clearly positive, although it has run over into the negative control as well, making it look like the negative control is also positive when it is not.

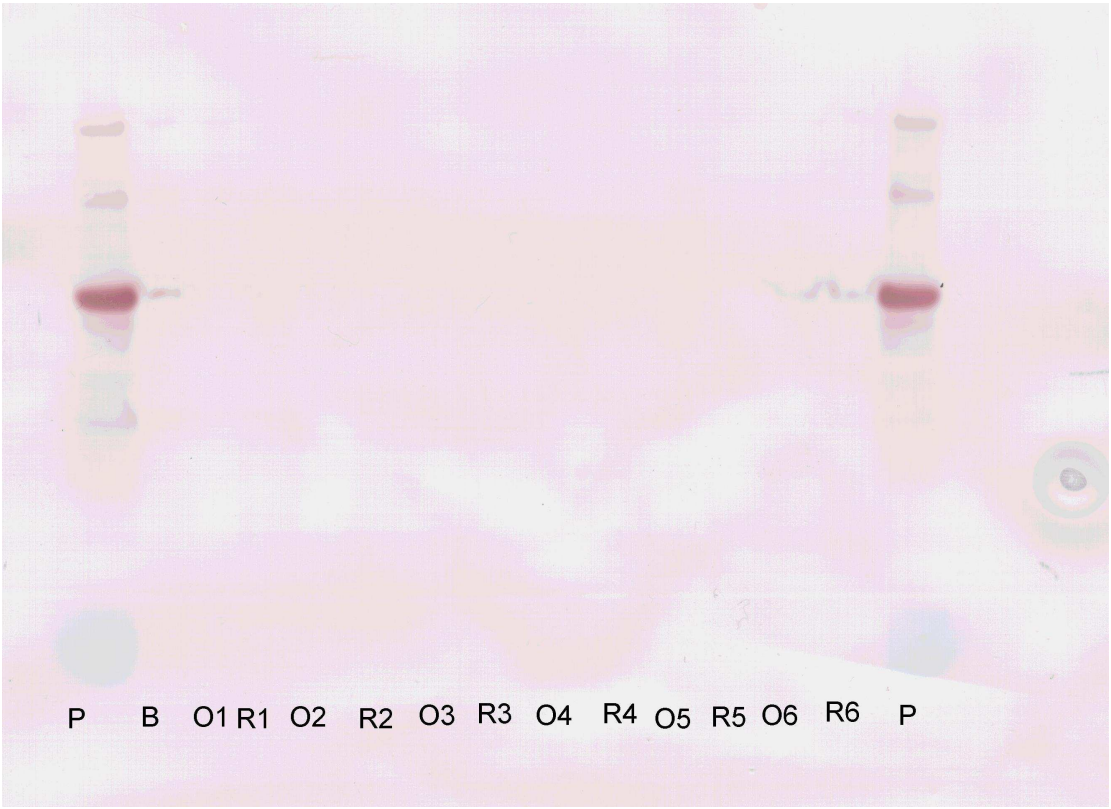


Figure 3.6: Colloidal Gold stains of strains A. g 13 TU 4793, A. g 13 TU 4799, K.p. 4841, K..p 4844, E.c. 4842, E.c. 4843

Legend: O = original extraction, R = re-extracted LPS, B = blank the negative control,
1-A. g 13 TU 4793, 2-A. g 13 TU 4799, 3-K.p. 4841, 4-K.p. 4844, 5-E.c. 4842, 6-E.c. 4843, P – positive control Mark 12 protein marker (Sigma)

As can be seen in Figure 3.6, the two positive controls both show that protein is present, whereas none of the LPS extractions had any protein in them and so it is possible to assume that they are pure of protein contamination.

3.1.4 Limulus Amoebocyte Lysate assay of the LPS extractions

The Limulus Amoebocyte Lysate assay uses lysate extracted from the amoebocytes of *Limulus polyphemus*, the horseshoe crab. This lysate gels when it comes into contact with bacterial endotoxin (Lindsey *et al*, 1989). This gelation means that it can be used as an assay for measuring how endotoxic a particular LPS is. This is carried out by making a number of 10-fold dilutions on a plate and measuring how turbid they make the Lysate and then comparing this to a standard curve of LPS with known endotoxicity levels. It is thus possible to measure the endotoxicity units of an unknown LPS. All the results in Figure 3.7 below were carried out using re-extracted LPS and were duplicates.

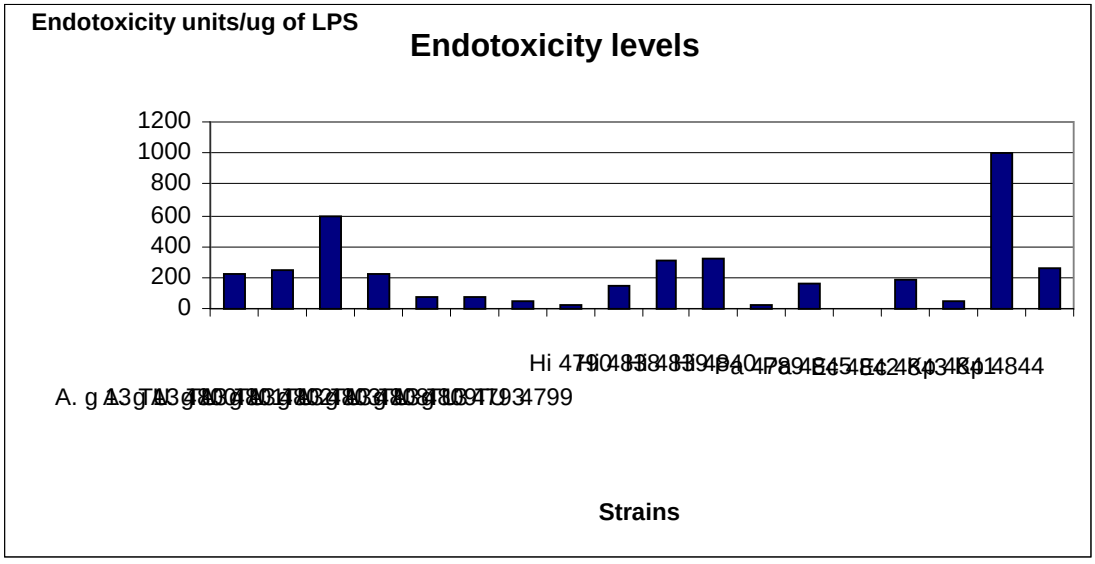


Figure 3.7: The Endotoxicity levels of the re-extracted LPS from the bacterial strains studied.

Figure 3.7 shows the endotoxicity levels of the LPS from the bacterial strains. Half of the strains were around the 200 endotoxicity units/µg mark. Seven strains were below 100 endotoxicity units/µg. Two strains however, *Acinetobacter* genospecies 13 TU 4802 and *Klebsiella pneumoniae* 4841 were 600 and 1000 endotoxicity units/µg

respectively. This shows that the majority of the strains tested had low endotoxicities, but that two of the strains had very high endotoxicities and thus were more likely to be more aggressive pathogens.

3.2 Investigating how widespread antibodies to *Acinetobacter* genospecies 13 TU and *Haemophilus influenzae* Lipopolysaccharide are in the healthy population of Southeast Scotland

3.2.1 Antibodies to *Acinetobacter* genospecies 13 TU LPS in the healthy population of Southeast Scotland

3.2.1.1 ELISA results for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU strains.

Serum was isolated from 475 blood samples, given by blood donors to the Blood Transfusion Service of Southeast Scotland. The levels of antibodies in these blood donor samples to the LPS from eight different strains of *A.* genospecies 13 TU were measured by ELISA, following 100-fold dilution of the serum and reaction with the LPS-polymyxin complexes coated at 5μg/ml on a 96 well Nunc Medisorp plate. The results are summarised in figures 3.8-3.16.

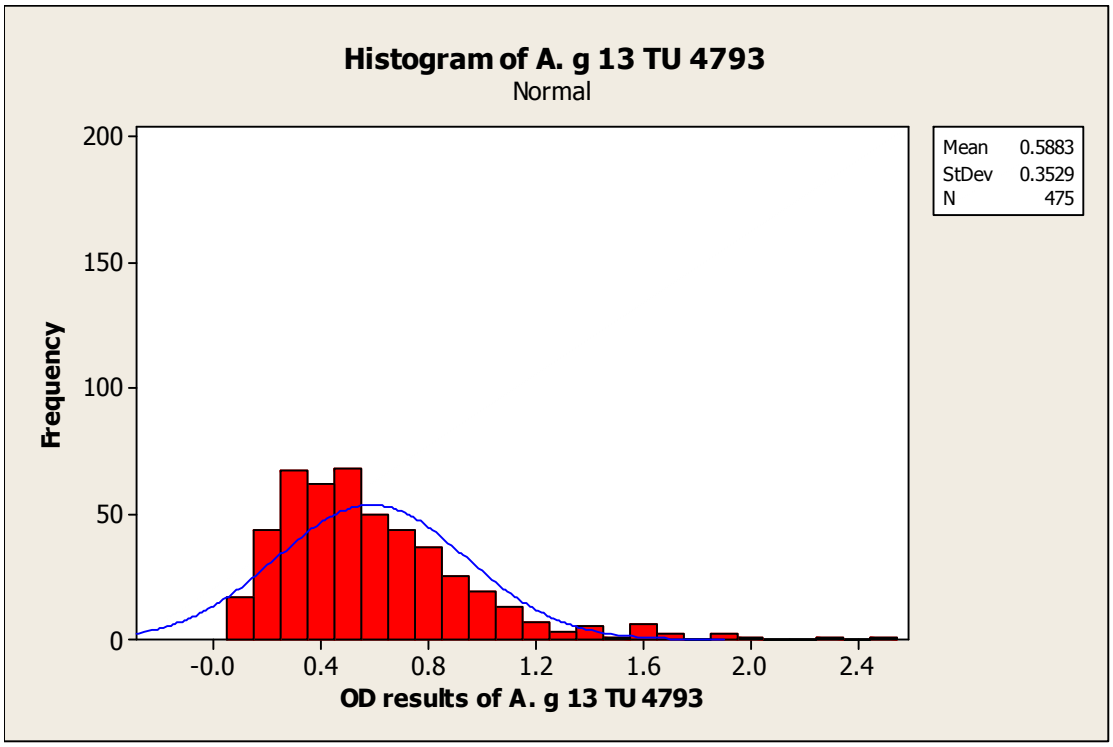


Figure 3.8: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4793. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4793 in the range indicated on the horizontal axis.

In Figure 3.8 the mean optical density (OD) range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4793 of 0.588 is greater when compared with several of the subsequently analyzed strains. Also the normal curve is very flat with a large standard deviation of 0.3529. All of this shows that the antibody levels in a healthy population of Southeast Scotland to *Acinetobacter* genospecies 13 TU 4793 LPS are larger than those of some of the subsequently analyzed strains. The reason it is possible to say this is that with a larger mean and a higher standard deviation these indicate a greater frequency of high responders in the population, which partially correlates with how often the population has been exposed to the LPS organism.

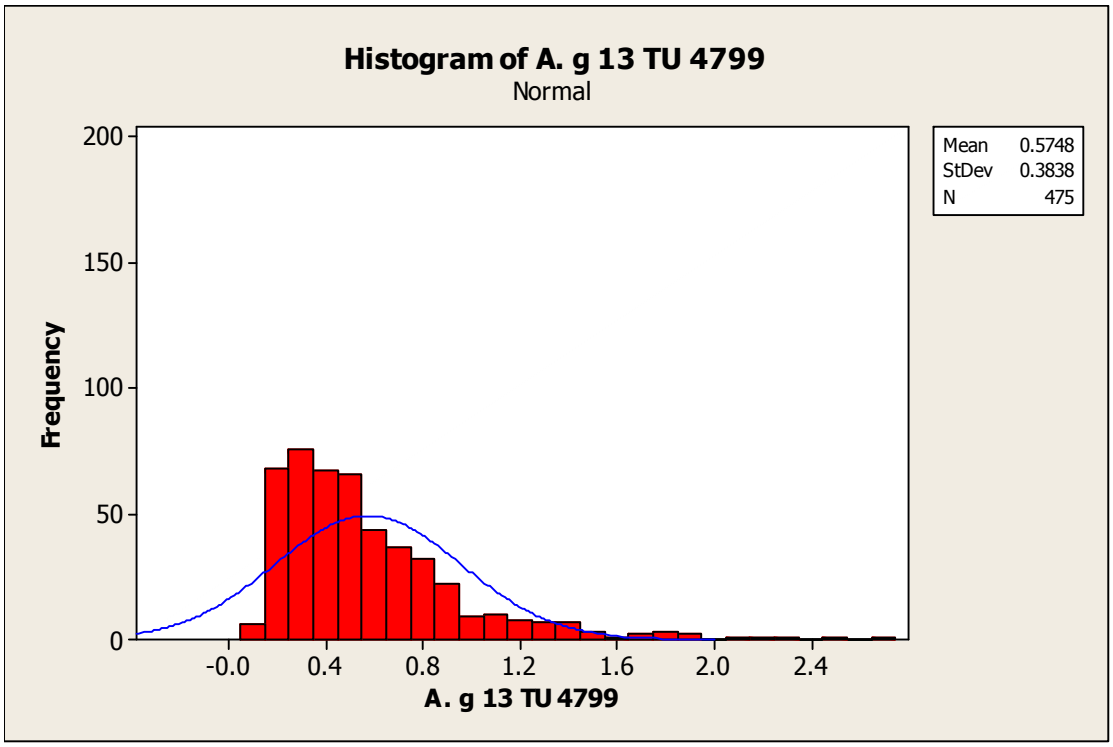


Figure 3.9: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4799. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4799 in the range indicated on the horizontal axis.

In Figure 3.9 the mean OD range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4799 of 0.5748 is very similar to those of *Acinetobacter* genospecies 13 TU 4793. The normal curve is very flat, with a similar standard deviation of 0.3838 to that of *Acinetobacter* genospecies 13 TU 4793 as shown in Figure 3.8. All of this indicates that exposure to *Acinetobacter* genospecies 13 TU 4799 LPS in a healthy population of Southeast Scotland is very similar to that of *Acinetobacter* genospecies 13 TU 4793 LPS.

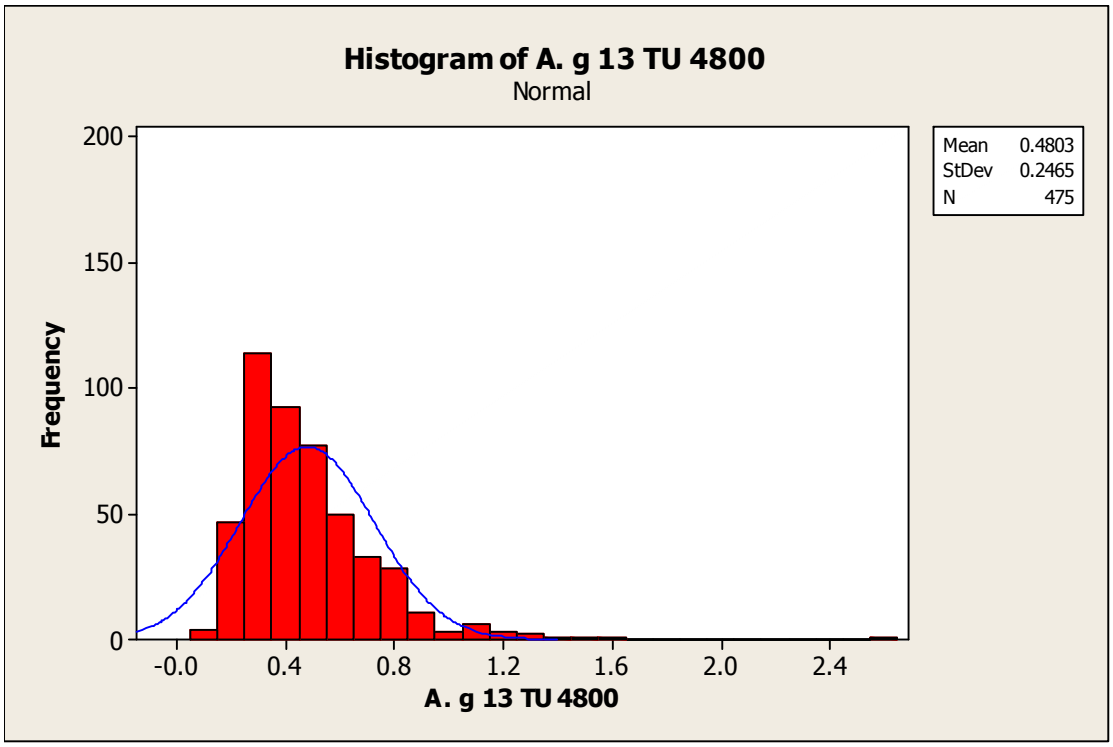


Figure 3.10: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4800. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4800 in the range indicated on the horizontal axis.

In Figure 3.10 the mean OD range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4800 of 0.4803 is lower than that of *Acinetobacter* genospecies 13 TU 4793 and *Acinetobacter* genospecies 13 TU 4799. The normal curve is more pronounced, with a smaller standard deviation of 0.2465 than those of *Acinetobacter* genospecies 13 TU 4793 and *Acinetobacter* genospecies 13 TU 4799. All of this shows that exposure to *Acinetobacter* genospecies 13 TU 4800 LPS in a healthy population of Southeast Scotland is at a lower level than those of the two previous strains of *Acinetobacter* genospecies 13 TU.

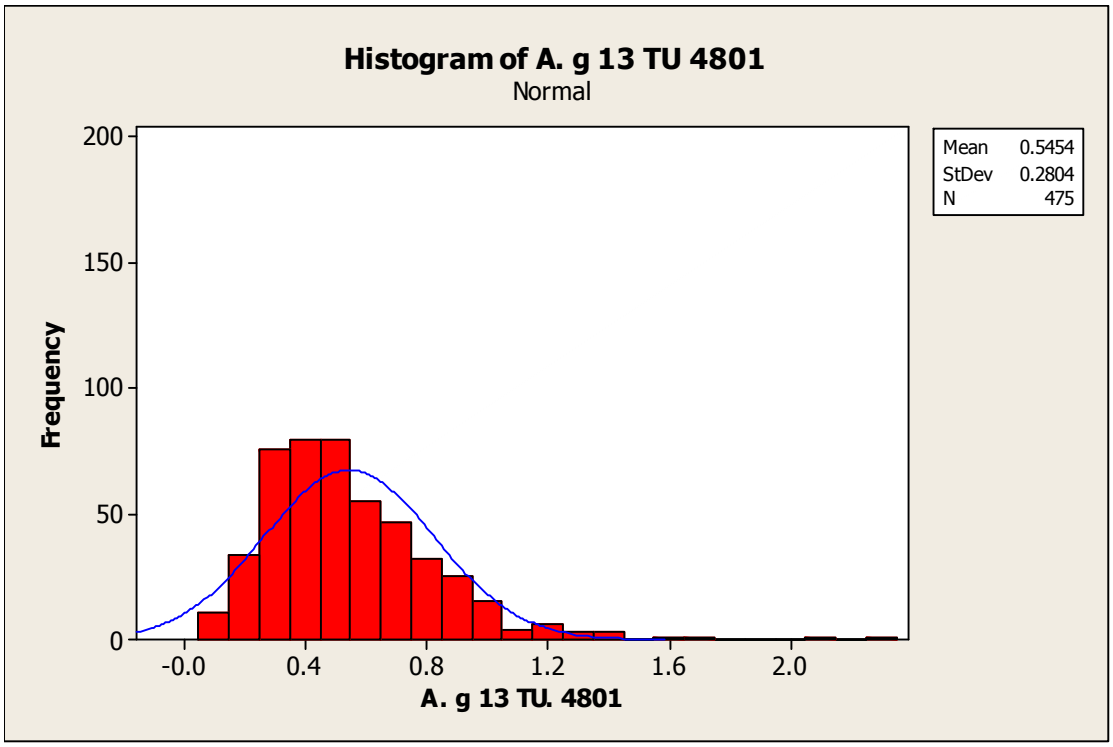


Figure 3.11: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4801. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4801 in the range indicated on the horizontal axis.

In Figure 3.11 the mean OD range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4799 of 0.5454 is very similar to those of *Acinetobacter* genospecies 13 TU 4793 and *Acinetobacter* genospecies 13 TU 4801. The normal curve is very flat, with a similar standard deviation of 2.804, which is less than that of *Acinetobacter* genospecies 13 TU 4793 and *Acinetobacter* genospecies 13 TU 4799, but greater than that of *Acinetobacter* genospecies 13 TU 4800. This means that there is less variety of results and they are more tightly grouped around the mean. All of this indicates that exposure to *Acinetobacter* genospecies 13 TU 4801 LPS in a healthy population of Southeast Scotland is similar to, but slightly less than the exposure levels to *Acinetobacter* genospecies 13 TU 4793 and *Acinetobacter* genospecies 13 TU 4799 LPS.

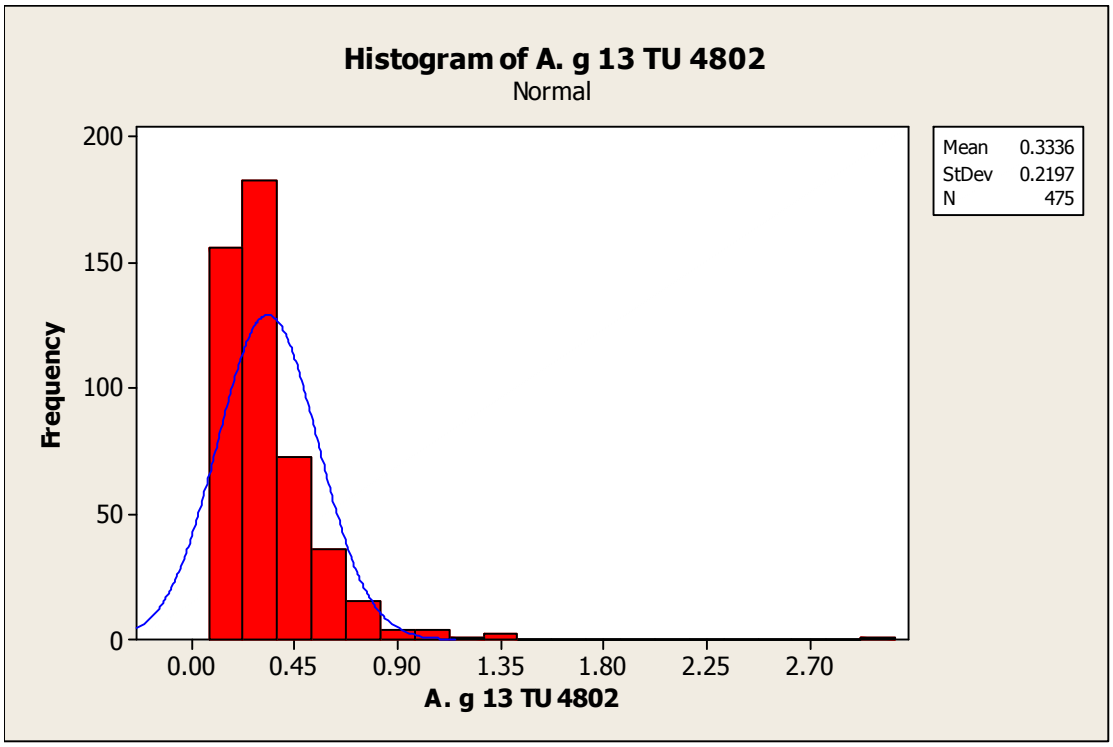


Figure 3.12: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4802. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4802 in the range indicated on the horizontal axis.

In Figure 3.12 the mean OD range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4802 of 0.3336 is lower than all of the previously described *Acinetobacter* genospecies 13 TU strains. The normal curve is much more pronounced, with a standard deviation of 0.2197, which whilst smaller than those of *Acinetobacter* genospecies 13 TU 4793 and *Acinetobacter* genospecies 13 TU 4799, was closer to that of *Acinetobacter* genospecies 13 TU 4800. All of this shows that exposure to *Acinetobacter* genospecies 13 TU 4802 LPS in a healthy population of Southeast Scotland is at a lower level than in all the previously described strains of *Acinetobacter* genospecies 13 TU.

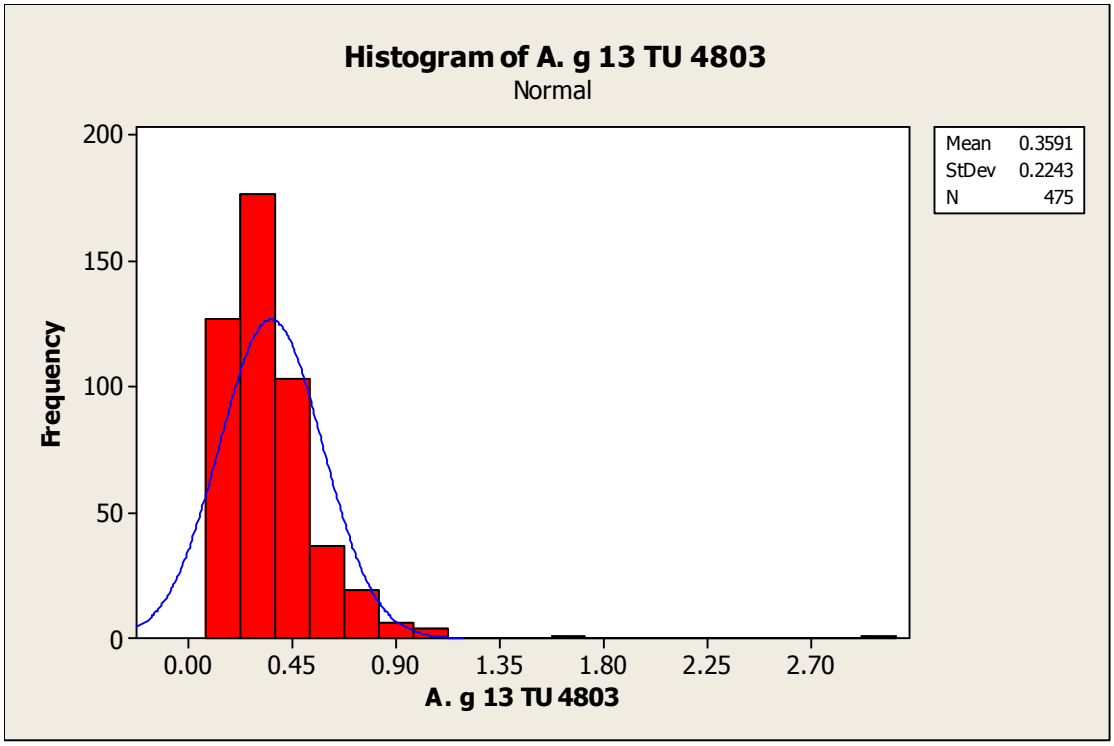


Figure 3.13: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4803. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4803 in the range indicated on the horizontal axis.

In Figure 3.13 the mean OD range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4803 of 0.3591 is lower than most of the previously described *Acinetobacter* genospecies 13 TU strains. The normal curve and the standard deviation of 0.2243 are very close to the normal curve and standard deviation of *Acinetobacter* genospecies 13 TU 4802. Thus the level of exposure in the healthy population of Southeast Scotland to *Acinetobacter* genospecies 13 TU 4803 LPS is very similar to that of *Acinetobacter* genospecies 13 TU 4802 LPS. Thus these two *Acinetobacter* genospecies 13 TU strains seem to have lower exposure levels to their LPS than the other strains tested.

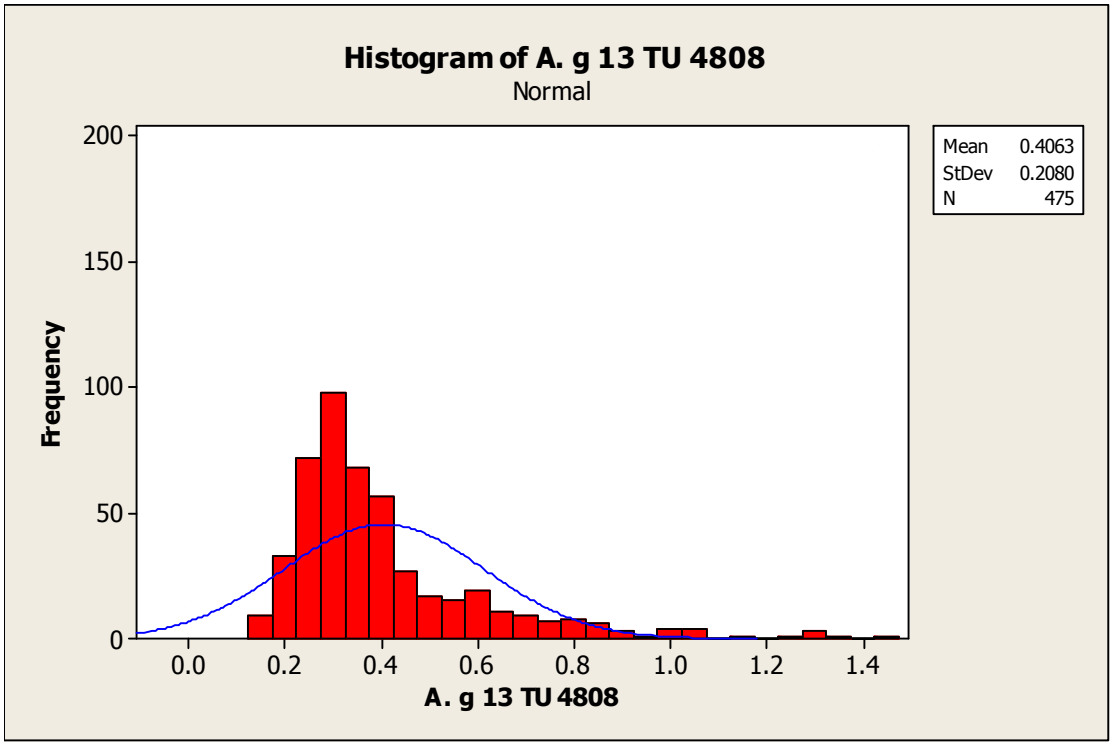


Figure 3.14: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4808. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4808 in the range indicated on the horizontal axis.

In Figure 3.14 the mean OD range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4808 of 0.4063 is lower than that of *Acinetobacter* genospecies 13 TU 4800, but greater than those of *Acinetobacter* genospecies 13 TU 4802 and *Acinetobacter* genospecies 13 TU 4803. The normal curve is flatter than thse of *Acinetobacter* genospecies 13 TU 4802 and *Acinetobacter* genospecies 13 TU 4803, but has a smaller standard deviation of 0.208 than either of the two strains just mentioned. All of this shows that exposure to *Acinetobacter* genospecies 13 TU 4808 LPS in a healthy population of Southeast Scotland is at a lower level than *Acinetobacter* genospecies 13 TU 4800 LPS, but higher than *Acinetobacter* genospecies 13 TU 4802 LPS and *Acinetobacter* genospecies 13 TU 4803 LPS.

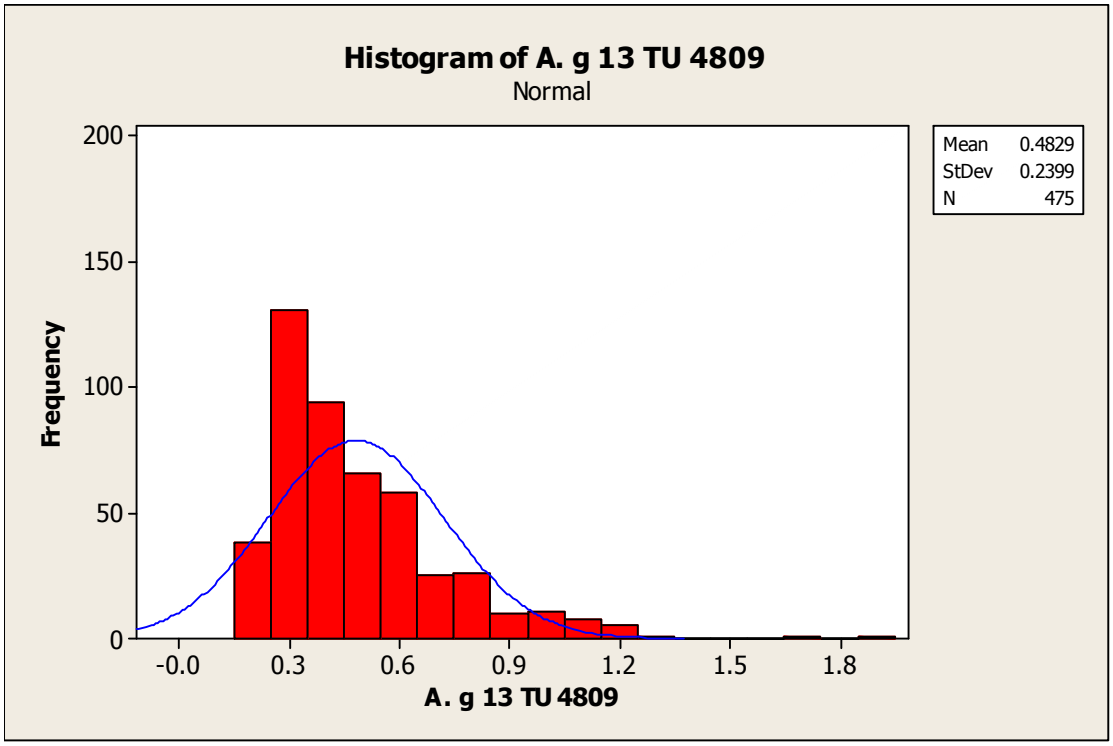


Figure 3.15: Histogram of the frequency of OD results from ELISAs with the LPS of *Acinetobacter* genospecies 13 TU 4809. Frequency is the number of individual blood donors with a particular antibody level to *Acinetobacter* genospecies 13 TU 4809 in the range indicated on the horizontal axis.

In Figure 3.15 the mean OD range for antibody levels to the LPS of *Acinetobacter* genospecies 13 TU 4809 of 0.4829 is very similar to that of *Acinetobacter* genospecies 13 TU 4800. The normal curve and the standard deviation of 0.2399 are also very similar to those of *Acinetobacter* genospecies 13 TU 4800. Thus exposure to *Acinetobacter* genospecies 13 TU 4809 LPS in a healthy population of Southeast Scotland is at a similar level to exposure to *Acinetobacter* genospecies 13 TU 4800 LPS.

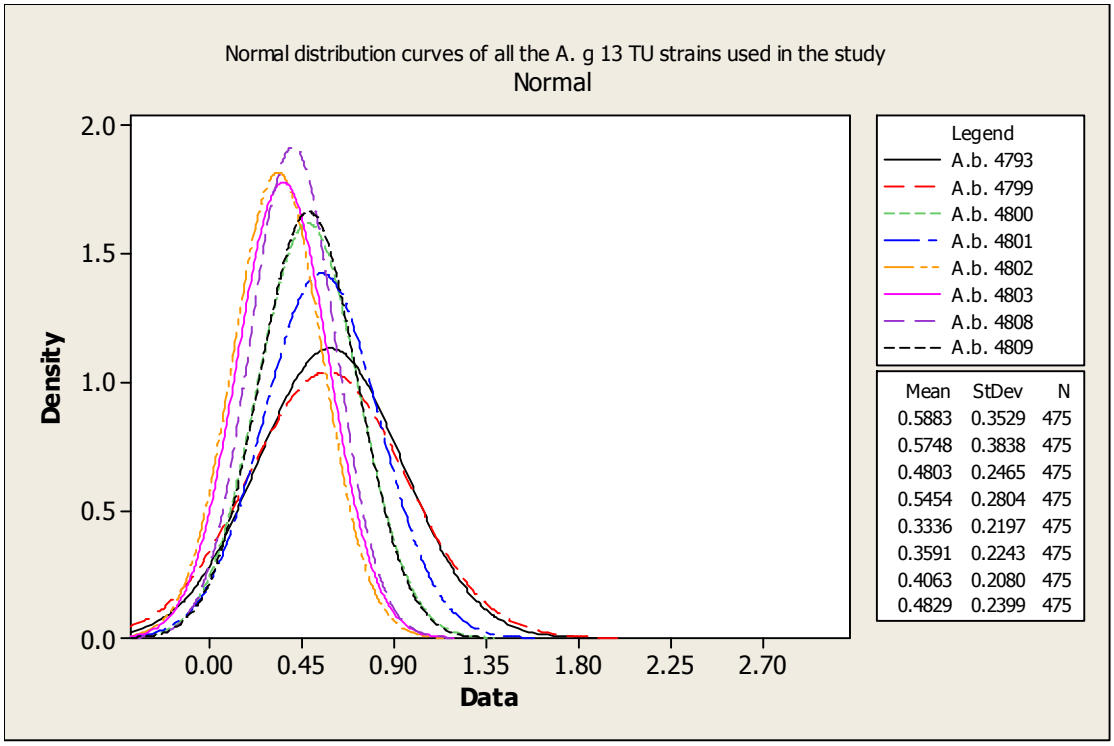


Figure 3.16: All the normal curves from the antibody levels to the LPSs of the eight *Acinetobacter* genospecies 13 TU strains placed on a graph together. The graph has all the normal curves of the eight *Acinetobacter* genospecies 13 TU strains with their means and standard deviations.

As can be seen in Figure 3.16, there is a wide variety of different normal curves with some being taller and narrower than others. There is also a variety of the wider lower normal curves seen in the figure. The reason that some of the normal curves are high and narrow is that the antibody levels to the LPS from those strains were much more grouped about the mean than for other strains. This leads to a greater frequency of these results and thus the normal curves are much higher and narrower. The wider, flatter normal curves have a greater distribution of results and thus have a lower frequency of certain results appearing, so the curves are wider and flatter. The normal curves when taken in conjunction with the means show that the antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU 4793 and *Acinetobacter genospecies 13 TU* 4799 are very similar in their distribution and means, which implies that exposure

to them is similarly widespread in the healthy population of Southeast Scotland. The results of antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU 4800 and 4809 also have very similar normal curves and means, implying that there is a similar level of exposure to them across the healthy population of Southeast Scotland. Levels to the LPSs of *Acinetobacter* genospecies 13 TU 4802, 4803 and to a lesser extent 4808 all have similar means and shapes of their normal curves, which again indicates that the exposure levels to these strains are similar in the healthy population of Southeast Scotland. The antibody levels to *Acinetobacter* genospecies 13 TU 4801 LPS have a higher mean than those against *Acinetobacter* genospecies 13 TU 4802, 4803, 4808, 4800 and 4809 LPSs, but are lower than those of *Acinetobacter* genospecies 13 TU 4793 and 4799 LPSs. So they are at the higher end of the exposure levels to these eight *Acinetobacter* genospecies 13 *TU* strains but not the highest.

3.2.1.2 Comparison of results from *Acinetobacter* genospecies 13 TU with other organisms.

The ELISA results of antibody levels from each serum specimen to LPSs were averaged across all the strains tested within a species. These averaged OD results were plotted in scatterplots against the averaged OD results of other species with well-characterised LPSs, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The means of these antibody levels to the LPSs of the different species were also included on the scatterplots and compared with each other. The first two species are common human gut commensals that are often also opportunistic pathogens. The last organism is a common environmental organism that is also a common opportunistic pathogen. The antibody levels are indicative of the level of exposure that the population has had to the LPS of this organism; thus it is possible to

draw conclusions as to how widespread exposure to *Acinetobacter* genospecies 13 TU is in comparison to the other organisms in the healthy population of Southeast Scotland.

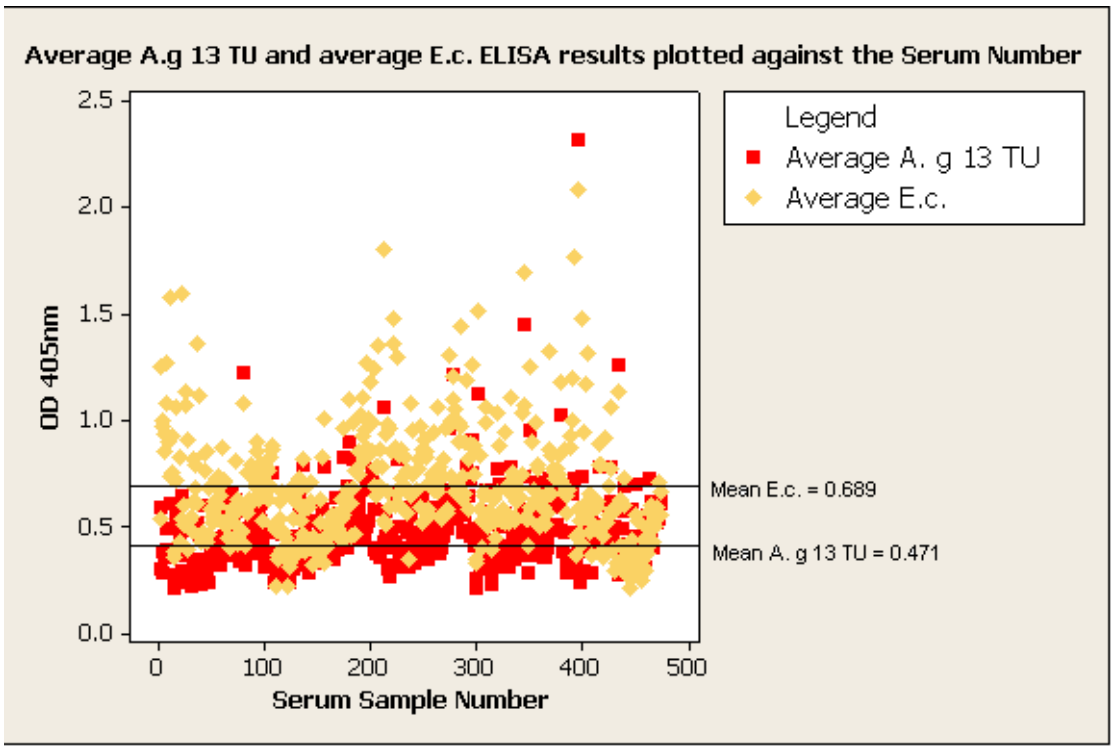


Figure 3.17: Scatter plot of the averaged OD results of antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU and *E. coli* against the serum sample number. The means of the averaged OD results are also plotted on the graph.

As can be seen in Figure 3.17, the mean of the antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU are significantly ($p<0.001$) lower than the mean of *E. coli* LPS and thus the spread of the responses to the lipopolysaccharides is very much higher for *E. coli*.

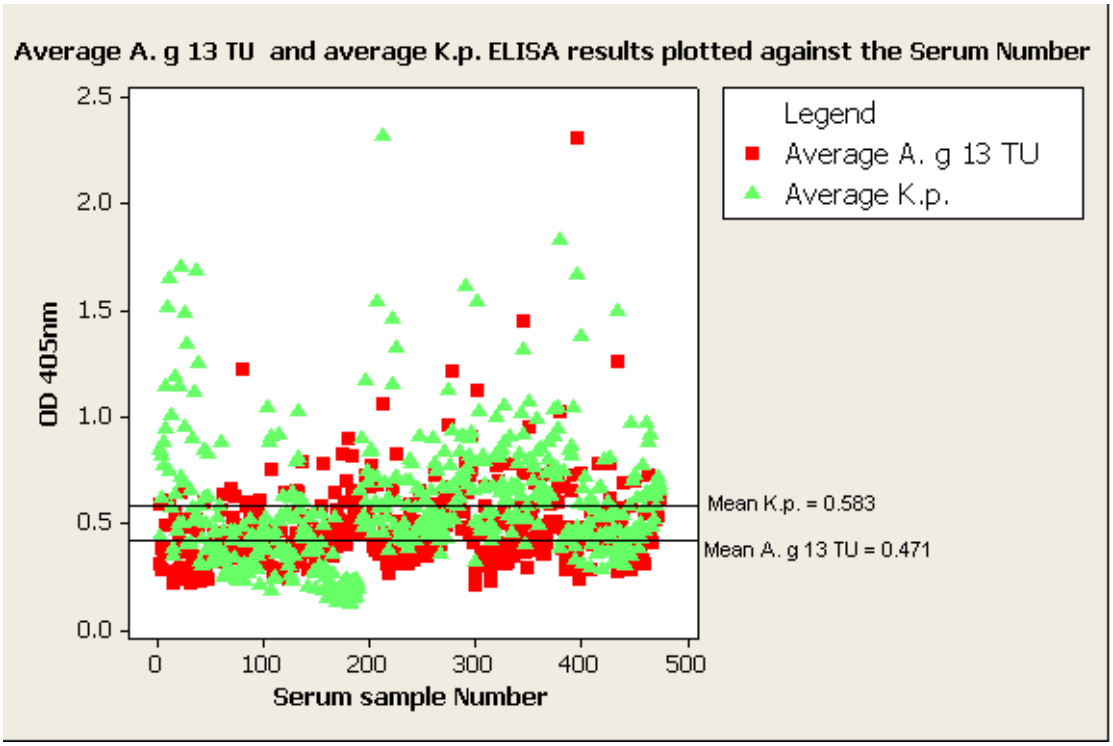


Figure 3.18: Scatter plot of the averaged OD results of antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU and *Klebsiella pneumoniae* against the serum sample number. The means of the averaged OD results are also plotted on the graph.

As Figure 3.18 shows, the mean of the antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU is significantly ($p<0.001$) smaller than the mean of *Klebsiella pneumoniae*, but the means are much closer to each other then the mean of *Acinetobacter* genospecies 13 TU is to the mean of *E. coli*. When the points on the graph are compared, it is possible to see that *Klebsiella pneumoniae* LPS has a greater number of higher responders, but also has a greater number of lower responders, which is why the means are that much closer.

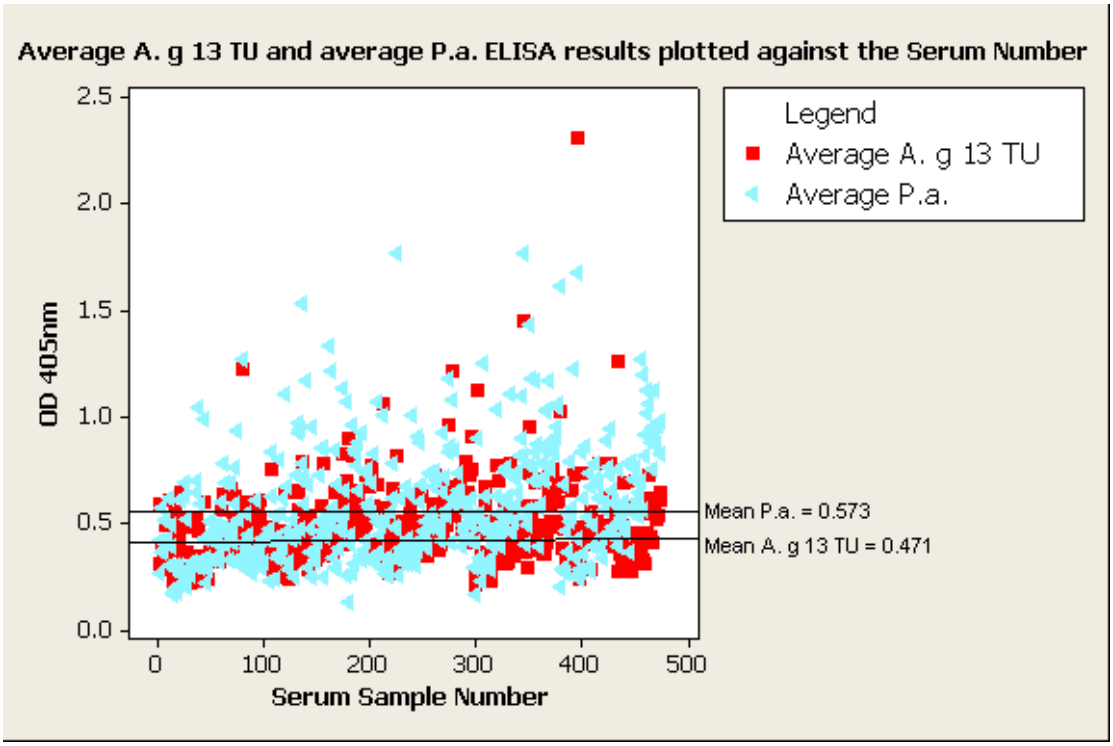


Figure 3.19: Scatter plot of the averaged OD results of antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU and *Pseudomonas pneumoniae* against the serum sample number. The means of the averaged OD results are also plotted on the graph.

Figure 3.19 demonstrates that, whilst the mean of antibody levels to the LPSs of *Acinetobacter* genospecies 13 TU is significantly ($p<0.001$) smaller than that of *Pseudomonas aeruginosa* LPS, it is a smaller difference than between the means of antibody levels to the LPSs of *Klebsiella pneumoniae* and *Acinetobacter* genospecies 13 TU. When the points on the graph are compared, it is possible to see that antibody levels to the LPSs *Pseudomonas aeruginosa* and *Acinetobacter* genospecies 13 TU are very closely overlaid on top of each other indicating that the antibody response to the two of them is very close.

3.2.2 Antibodies to *Haemophilus influenzae* LPS in the healthy population of Southeast Scotland

3.2.2.1 ELISA results for antibody levels to the LPS of *Haemophilus influenzae* strains.

These experiments were carried out in exactly the same manner as for the *Acinetobacter* genospecies 13 TU strains. The results of the levels of antibodies to the LPS from four strains of *Haemophilus influenzae* were also compared with the levels of antibody to LPS from *E. coli*, *K. pneumoniae* and *P. aeruginosa*. This was done to allow conclusions to be drawn as to how widespread exposure to *Haemophilus influenzae* is in the healthy population of Southeast Scotland.

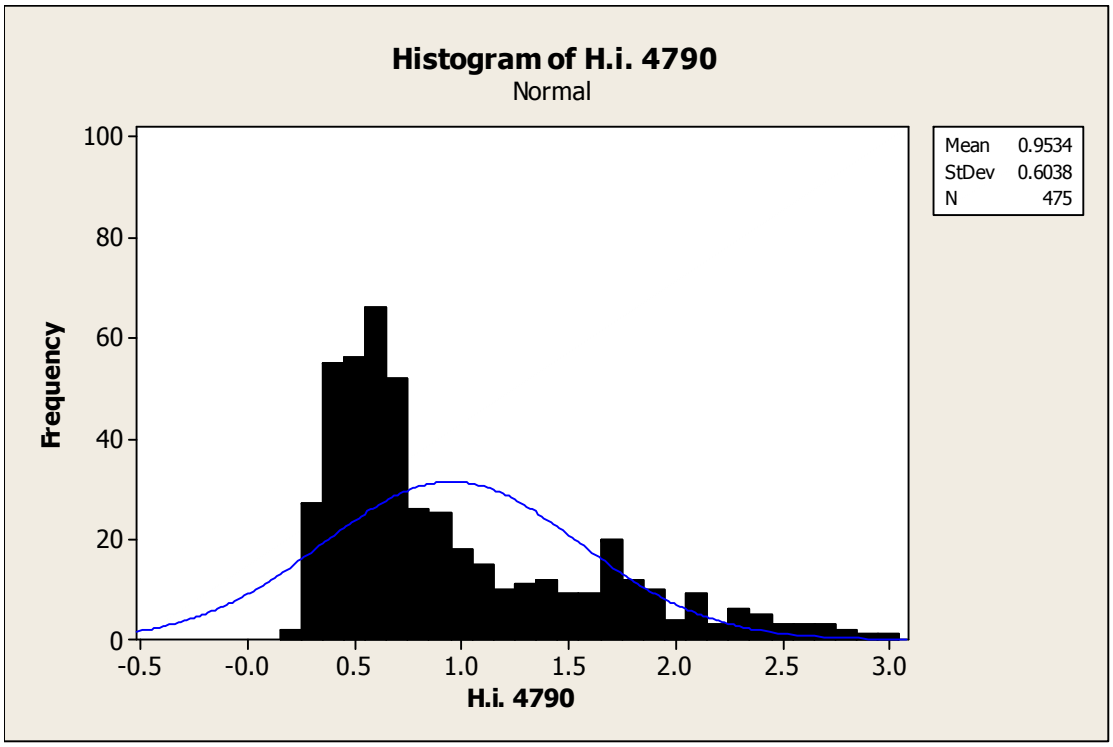


Figure 3.20: Histogram of the frequency of OD results from ELISAs with the LPS of *Haemophilus influenzae* 4790. Frequency is the number of individual blood donors with a particular antibody level to *Haemophilus influenzae* 4790 in the range indicated on the horizontal axis.

In Figure 3.20 the mean OD range for antibody levels to *Haemophilus influenzae* 4790 LPS of 0.9534. The normal curve is wide and flat and the standard deviation of 0.6038 is very large, giving the normal curve its wide and flat appearance. There is a possibility of bimodal distribution with a second peak around an OD of 1.7, but this is much lower, so it is harder to say. Thus it is possible say that exposure to *Haemophilus influenzae* 4790 LPS in a healthy population of Southeast Scotland is higher than all the other *Haemophilus influenzae* strains that were subsequently tested. The mean is much higher than those of the *Acinetobacter* genospecies 13 TU strains tested in Figures 3.8-3.15.

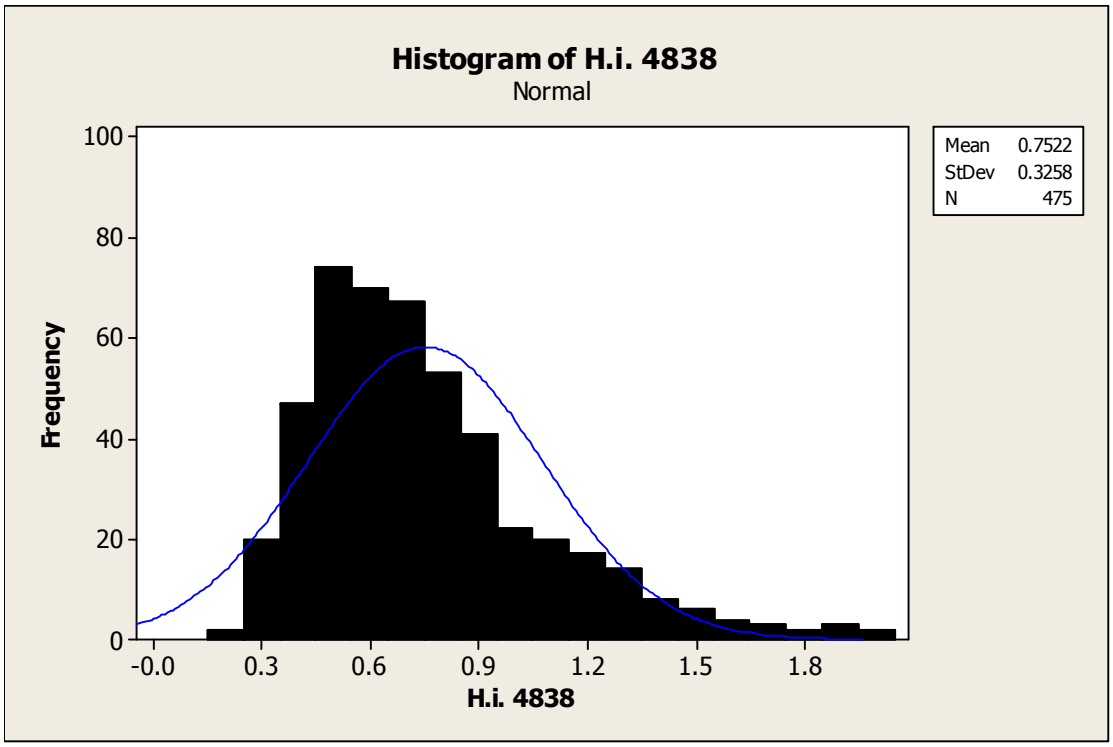


Figure 3.21: Histogram of the frequency of OD results from ELISAs with the LPS of *Haemophilus influenzae* 4838. Frequency is the number of individual blood donors with a particular antibody level to *Haemophilus influenzae* 4838 in the range indicated on the horizontal axis.

In Figure 3.21 the mean OD range for antibody levels to *Haemophilus influenzae* 4838 LPS of 0.7522; this mean is much lower than the mean of *Haemophilus influenzae* 4790. The normal curve is much more pronounced than with *Haemophilus influenzae* 4790 and the standard deviation of 0.3258 is much smaller, meaning that the data are much more closely grouped around the mean than in the previous figure. From the data presented in Figure 3.21 it is possible to say that exposure to *Haemophilus influenzae* 4838 LPS in a healthy population of Southeast Scotland is lower than that to *Haemophilus influenzae* 4790 LPS.

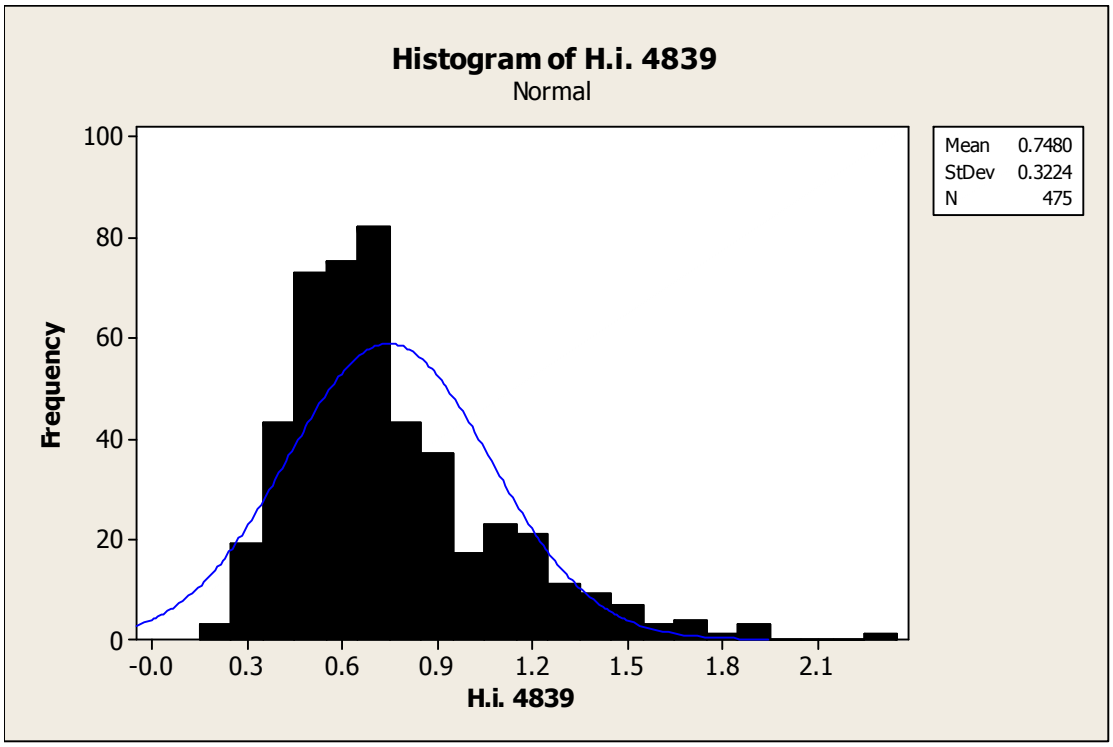


Figure 3.22: Histogram of the frequency of OD results from ELISAs with the LPS of *Haemophilus influenzae* 4839. Frequency is the number of individual blood donors with a particular antibody level to *Haemophilus influenzae* 4839 in the range indicated on the horizontal axis.

In Figure 3.22 the mean OD range for antibody levels to *Haemophilus influenzae* 4839 is 0.7480, which is very close to the mean of *Haemophilus influenzae* 4838. The normal curve is very similar to *Haemophilus influenzae* 4838 and the standard deviation of 0.3224 is also very close to that of *Haemophilus influenzae* 4838. Thus exposure to *Haemophilus influenzae* 4839 LPS in a healthy population of Southeast Scotland is very similar to the exposure to *Haemophilus influenzae* 4838 LPS.

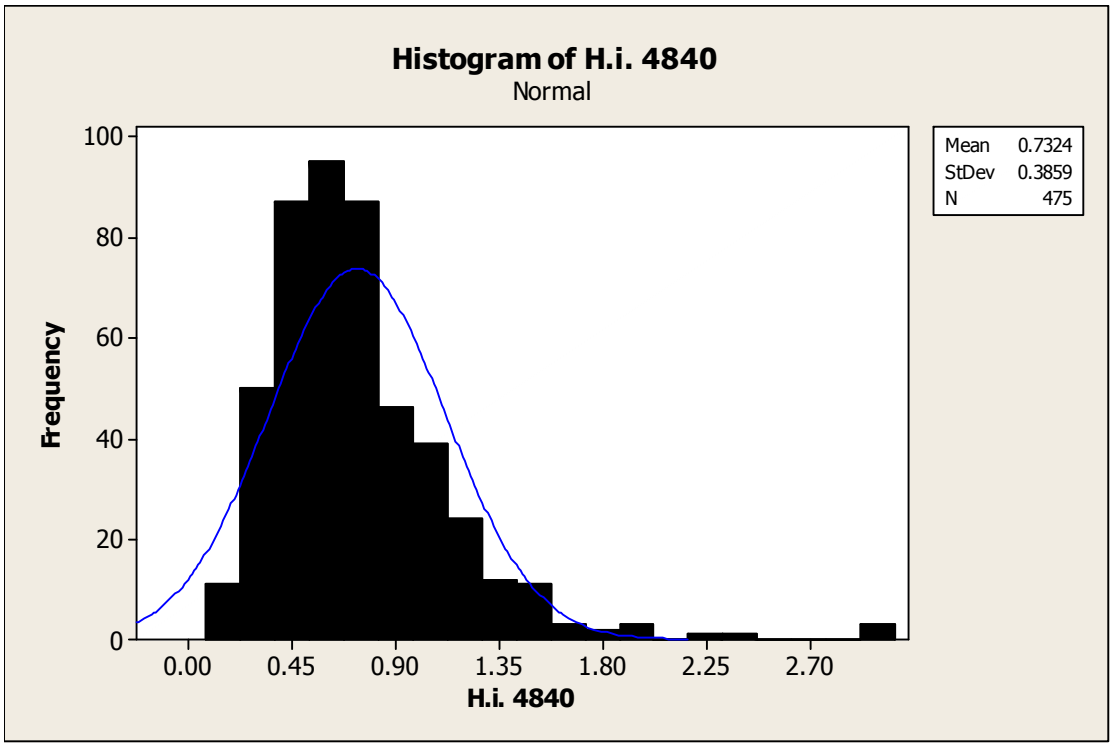


Figure 3.23: Histogram of the frequency of OD results from ELISAs with the LPS of *Haemophilus influenzae* 4839. Frequency is the number of individual blood donors with a particular antibody level to *Haemophilus influenzae* 4839 in the range indicated on the horizontal axis.

In Figure 3.23 the mean OD range for antibody levels to *Haemophilus influenzae* 4840 of 0.7324, this mean is very close to the means of *Haemophilus influenzae* 4838 and *Haemophilus influenzae* 4839. The normal curve and the standard deviation of 0.3859 are both very similar to *Haemophilus influenzae* 4838 and *Haemophilus influenzae* 4839. So it can be said exposure to *Haemophilus influenzae* 4840 LPS in a healthy population of Southeast Scotland is very similar to the exposure to both *Haemophilus influenzae* 4838 LPS and *Haemophilus influenzae* 4839.

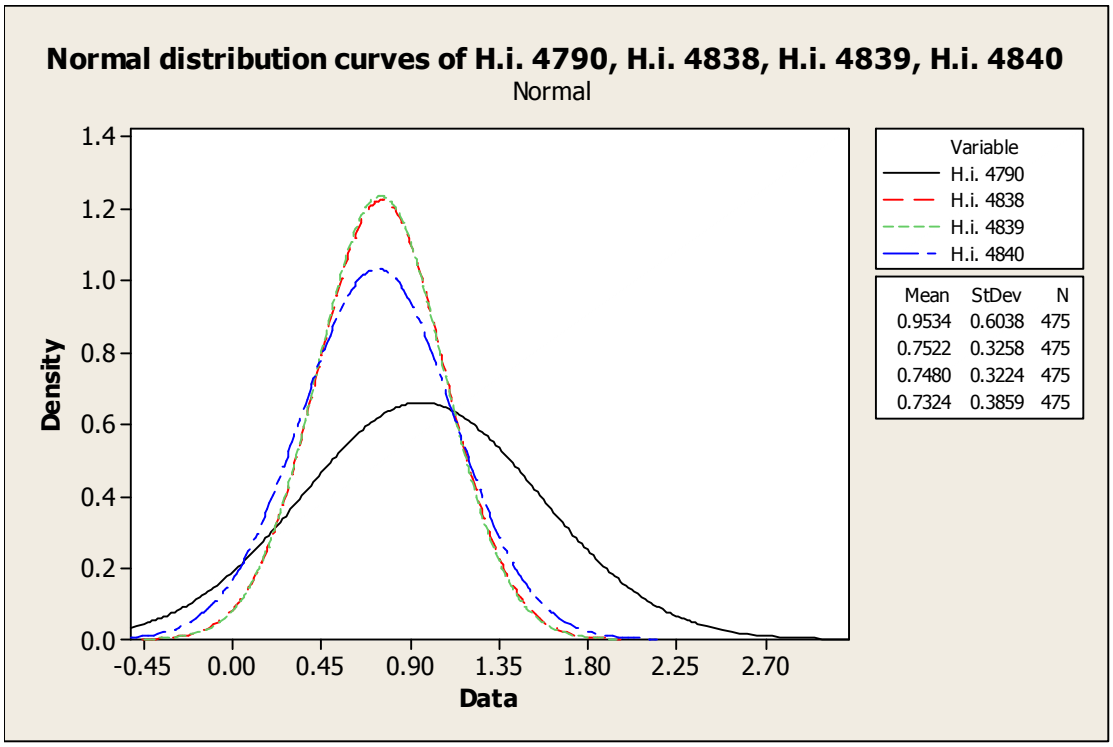


Figure 3.24: All the normal curves from the antibody levels to the LPSs of the four *Haemophilus influenzae* strains placed on a graph together. The graph has all the normal curves of the four *Haemophilus influenzae* strains with their means and standard deviations.

In Figure 3.24 there is a smaller amount of variation between the normal distribution curves than there was for the *Acinetobacter* genospecies 13 TU strains. This is partially due to the smaller group size, but as can be seen *Haemophilus influenzae* 4838, *Haemophilus influenzae* 4839 and *Haemophilus influenzae* 4840 have very similar distribution curves and also very similar means. The antibody levels to these three organisms can be said to be similar, since the distribution of the ELISA results is very close. *Haemophilus influenzae* 4790 LPS has a much higher mean and great distribution curve. So it seems that *Haemophilus influenzae* 4790 LPS antibody levels are higher than for the other three strains.

3.2.2.2 Comparison of Results from *Haemophilus influenzae* with other organisms.

As before, the ELISA results from across the strains tested were averaged within each species and their OD results were tested against some other well characterised species, namely *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. These were again plotted with scatterplots and the means were also compared. The antibody levels to the LPS of these organisms are indicative of the level of exposure that the population has had to that organism. From this it is possible to draw conclusions as to how widespread exposure to *Haemophilus influenzae* is in comparison to the other organisms in the healthy population of Southeast Scotland.

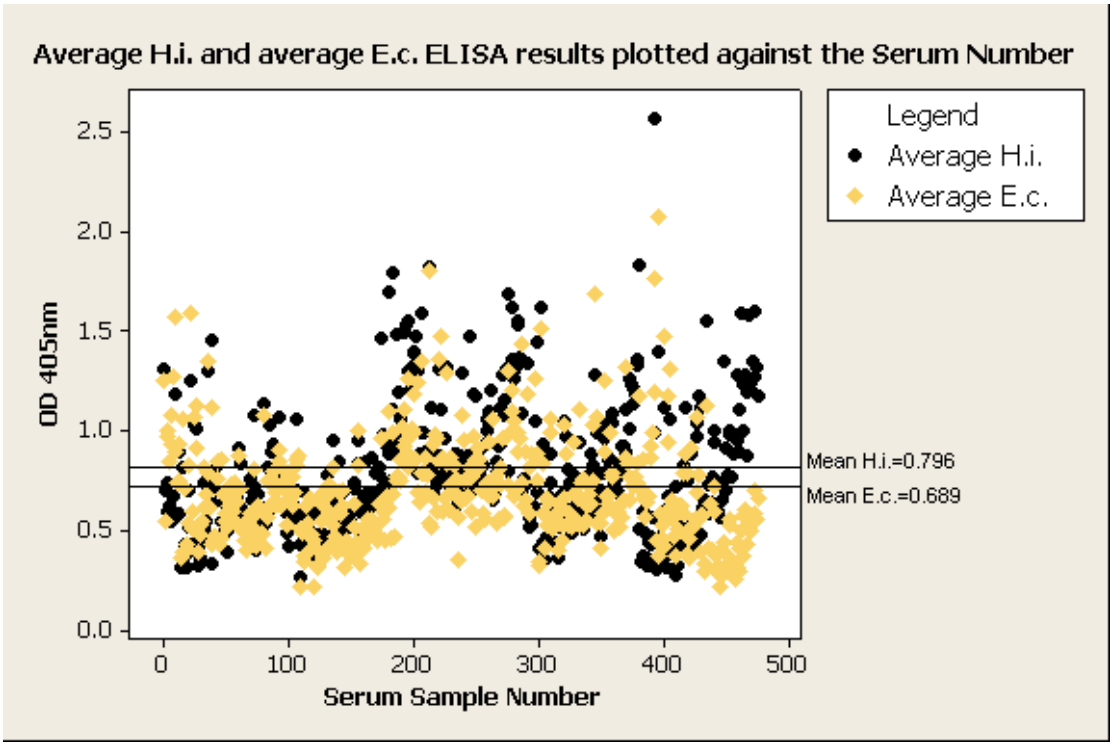


Figure 3.25: Scatter plot of the averaged OD results of *Haemophilus influenzae* and *E. coli* LPSs against the serum sample number. The means of the averaged OD results are also plotted on the graph.

In Figure 3.25 it can be seen that whilst the mean of *Haemophilus influenzae* LPS is significantly ($p<0.001$) larger than that of *E. coli* LPS, it is not massively so. Also the data plots of the two organisms can be seen to be overlying each other fairly closely indicating a similar level of antibodies found within the healthy population.

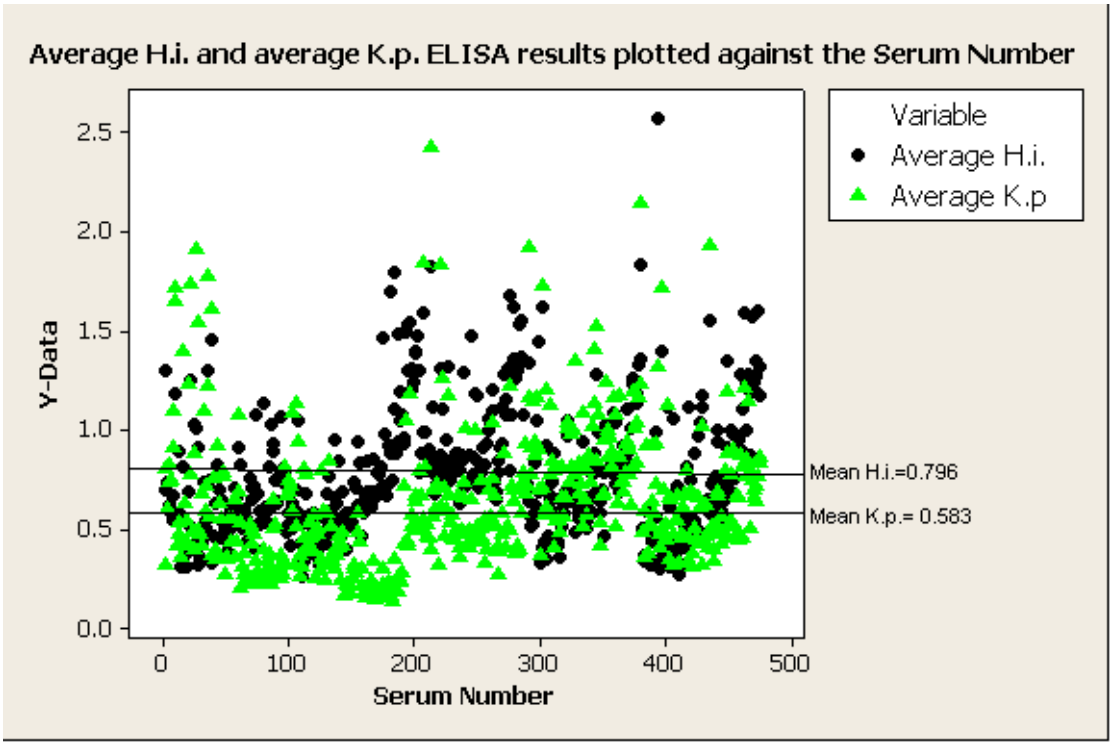


Figure 3.26: Scatter plot of the averaged OD results of *Haemophilus influenzae* and *Klebsiella pneumoniae* LPSs against the serum sample number. The means of the averaged OD results are also plotted on the graph.

As can be seen in Figure 3.26, the mean of *Haemophilus influenzae* LPS is significantly ($p<0.001$) greater than that of *Klebsiella pneumoniae* LPS. When the data plots of the two organisms are compared with each other, it is possible to see that *Klebsiella pneumoniae* LPS has much lower data points than *Haemophilus influenzae* LPS. This shows that antibody levels to *Haemophilus influenzae* LPSs are more widespread in the healthy population of Southeast Scotland than those of *Klebsiella pneumoniae* LPSs.

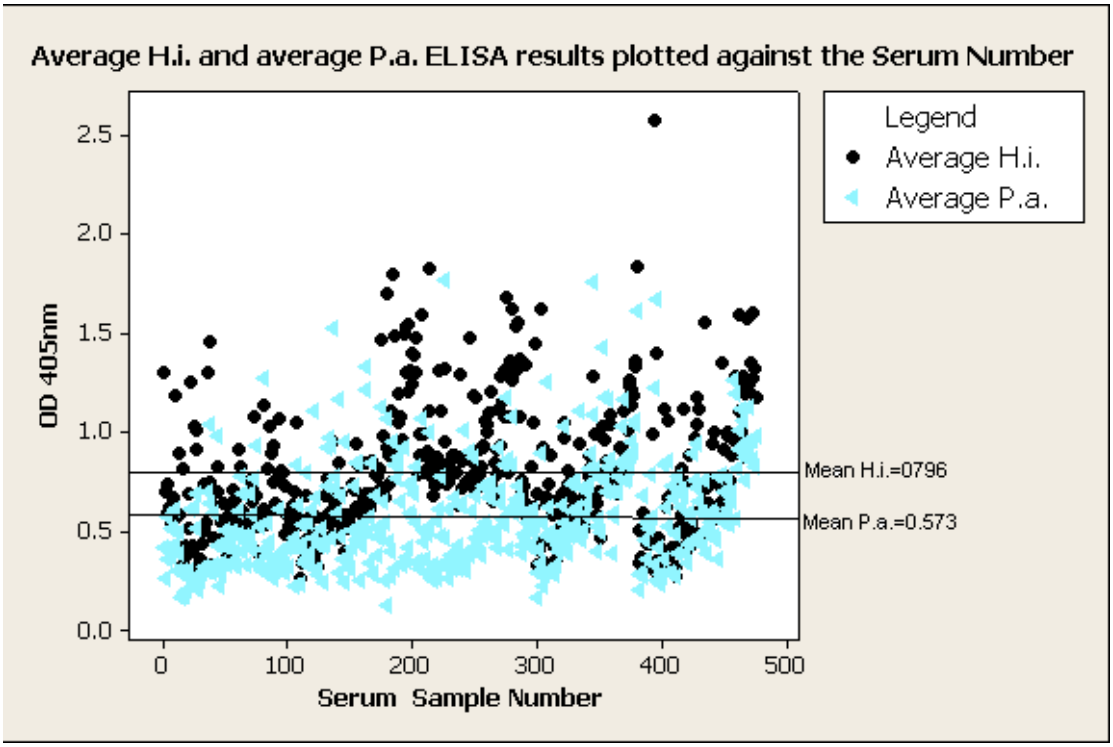


Figure 3.27: Scatter plot of the averaged OD results of *Haemophilus influenzae* and *Pseudomonas aeruginosa* LPSs against the serum sample number. The means of the averaged OD results are also plotted on the graph.

The results show that the mean of *Haemophilus influenzae* LPSs is significantly ($p<0.001$) greater than that of *Pseudomonas aeruginosa* LPSs. Comparison between the two data plots also shows that *Pseudomonas aeruginosa* LPSs has induced a much lower antibody response than *Haemophilus influenzae* LPSs. This shows that antibody levels to *Haemophilus influenzae* LPSs are more widespread in the healthy population of Southeast Scotland than those of *Pseudomonas aeruginosa* LPSs.

3.2.3 High and low responders in the healthy population of Southeast Scotland.

In a healthy population there is normally a wide range of different levels antibody responses to the LPS, but some people just have a lower reaction to any antigen they are challenged with. Others have consistently higher levels of antibody response. This can be seen in Figure 3.28.

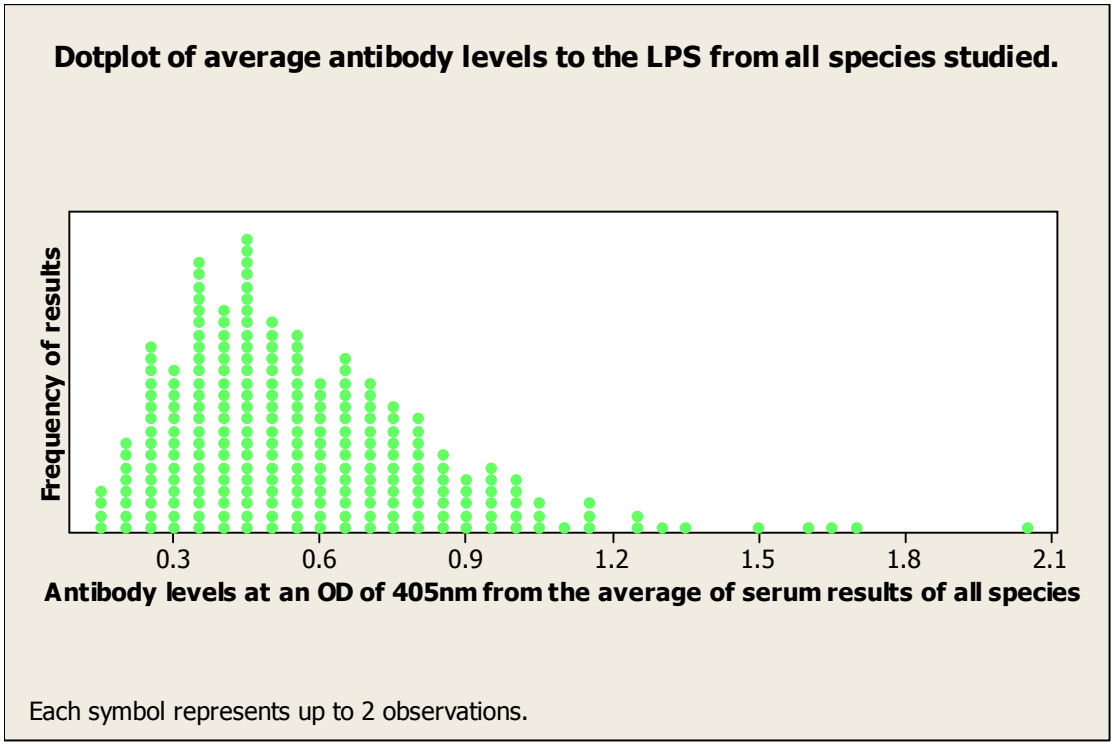


Figure 3.28: Dotplot of the average antibody level in the serum samples across all the LPS of the species studied. In this figure all the ELISA results for each serum sample were averaged out across the species and placed on this dotplot.

As can be seen in Figure 3.28 there are a wide range of possible antibody levels with the majority between an OD of 0.3 and 0.6. There are some individuals though that have a much higher level of response to all the different LPS. There are five individuals with average antibody levels across the LPS isolated from the 18 strains, which lie between an OD of 1.5 and 2.0. These are the high responders.

3.2.4 ELISA inhibition assays

These experiments were carried out to see if there was any cross-reactivity between any of the LPSs of the different strains. Since, if there were, it would mean that the ELISA results were likely to be due to reactions with the common regions of LPS and not to exposure to the strain-specific regions of the LPS of that strain. These experiments were to be carried out using inhibition assays as described below.

Serum samples from 250 of the blood donors were pooled together. A 96 well Nunc Medisorp plate was coated with a 1 in 8 dilution of LPS (equivalent to 0.125µg/ml) from one of the strains of interest. LPS from several strains including the LPS from the strain that was used to coat the plate were incubated for 30 minutes at 37ºC in a 1 in 50 dilution of the pooled serum at dilutions of: 1mg/ml, 200µg/ml, 40µg/ml, 8µg/ml, 1.6µg/ml, 0.32µg/ml, 0.064µg/ml and 0.013µg/ml. These were then treated as a normal ELISA and the inhibited serum samples were placed onto the plate in rows of each of the strains LPS dilutions. These were then read and the differences were measured.

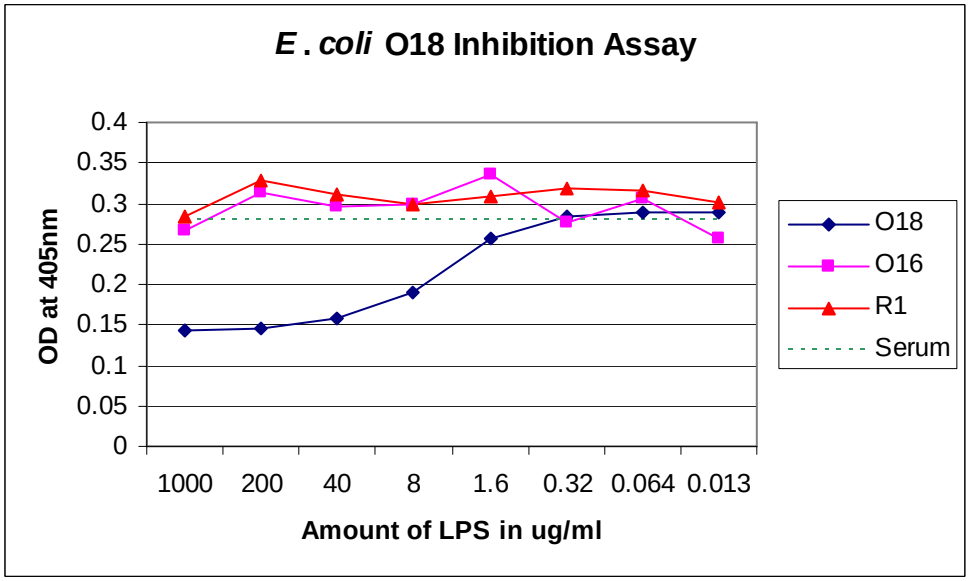


Figure 3.29: *E. coli* O18 inhibition assay. This experiment was carried out as a positive control with *E. coli* O18 to show that the technique worked. The optical densities of the results were read and put into the graph. The LPS was diluted in 8 5-fold dilutions.

As can be seen in Figure 3.29, at high concentrations the O18 free-floating LPS in the pooled serum inhibited the serum by binding to the free antibodies and thus reducing the amounts available. The other two *E. coli* LPSs which included a different O-type and a rough mutant had little or no inhibiting effect having the same results as the serum positive control. Thus it can be seen that the experiment works with *E. coli*. Unfortunately this technique does not appear to work with *Acinetobacter* genospecies 13 TU. This is shown in Figure 3.30 below.

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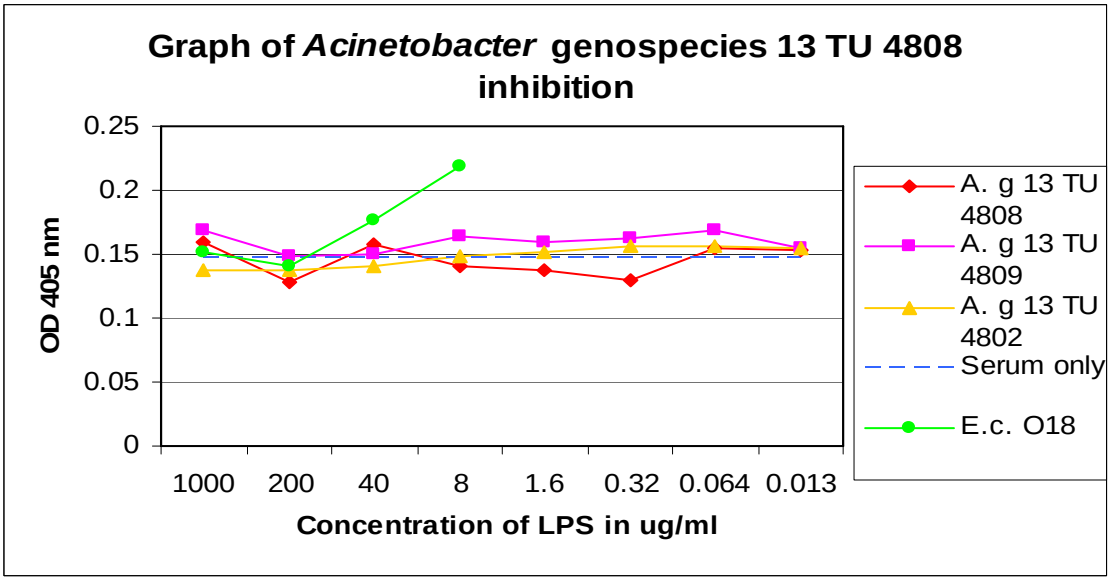

Figure 3.30: *Acinetobacter* genospecies 13 TU 4808 inhibition assay. The optical densities of the results were read and put into the graph. The LPS was diluted in 8 5-fold dilutions. *E. coli* O18 was included as a positive control.

In Figure 3.30, LPSs from three *Acinetobacter* genospecies 13 TU strains (including that from the coating strain) were used in an attempt to ascertain if there was any cross strain inhibition of LPS binding, which would show any structural similarities between the LPS of the different strains. As can be seen, there was no difference between the different *Acinetobacter* genospecies 13 TU strains including the coating and they lie on the serum-only positive control. This was included to show that the serum was in fact binding to the LPS bound to the plate. The *E. coli* O18 was also a positive control to show that the inhibition effect was working on the plate. The reason that it increases to above the serum only line is that it is an *E. coli* LPS and, as has been seen in the ELISA results, there is a greater amount of anti *E. coli* antibodies in the healthy population. So the above graph shows that there is no inhibition effect from any of the *Acinetobacter* genospecies 13 TU strains including the strain that was

coating the plate. Thus it seems that for some reason *Acinetobacter* genospecies 13 TU LPS in soluble form is unable to bind to the same antibodies as *Acinetobacter* genospecies 13 TU LPS that is bound to the Nunc Medisorp plate. This experiment was attempted with the same LPSs six times successfully and each time the results were the same. This situation was also observed when tested with LPS from other *Acinetobacter* genospecies 13 TU strains.

3.3 Results cytokine production induced by LPS with THP-1 studied by quantitative polymerase chain reaction (QPCR) experiments

3.3.1 Primer testing

The primers had to be tested to make sure that the products that they gave were correct. The testing was carried out using standard PCR. The primers were tested with DNA isolated from THP-1 cells. The PCR preperations were then run using the protocol as described in experiment 2.5.5. The products were run on a 2% agarose gel for 1.5 hours with a 100 base pairs (bp) ladder. The gel of the products can be seen in Figure 3.31.

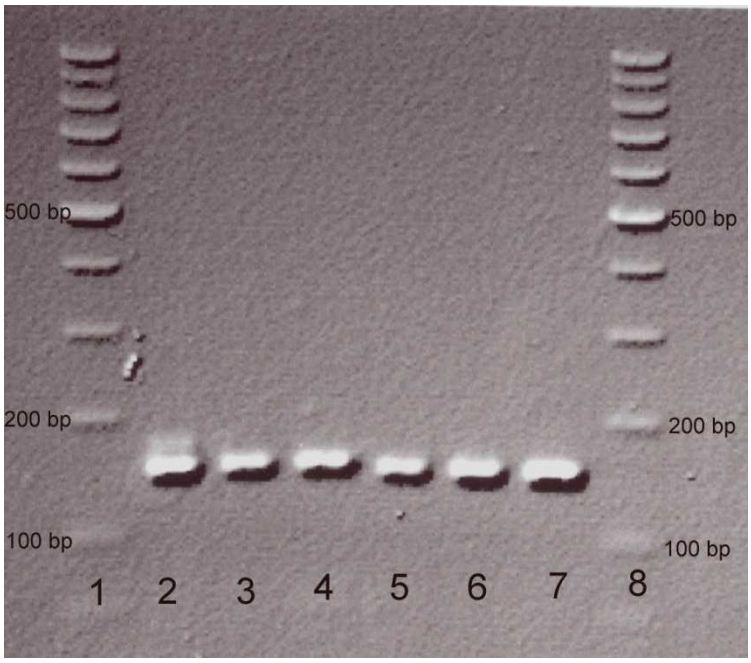

Figure 3.31: The primer tests of the six genes with the 100bp ladder.

1 - 100bp ladder, 2 - 18S ribosomal RNA, 3 - TNF-α, 4 - IFN γ, 5 - IL-1β, 6 - IL-8, 7 - IL-10, 8 - 100bp ladder.

As can be seen in Figure 3.31 the products of all the primers were the correct sizes. These were 151 bp for the 18S ribosomal RNA primers, 151bp for the TNF-α primers, 155bp for the IFN-γ primers, 151bp for the IL-1β primers, 151bp for the IL-8 primers and 150bp for the IL-10 primers. So it can been seen that the primers are all within 5bps of each other, as it is necessary for the products to be of nearly the same size when attempting to carry out QPCR.

3.3.2 Primer concentrations for QPCR

The optimal concentration of the primers had to be ascertained for the different genes. This was carried out by trying out four different concentrations of the primers: 50nM, 100nM, 200nM and400nM. These concentrations were run in a QPCR experiment as described in chapter 2.5.6.1 with THP-1 DNA, and the concentration that gave the

best response was chosen. The concentration that gave the best response for 18S ribosomal RNA, IFN-γ, IL-1β, IL-8 and IL-10 was the 200nM concentration. The best concentration for TNF-α was 400nM.

3.3.3 cDNA selection.

THP-1 cells had been grown and differentiated using vitamin D3 as described in chapter 2.4.2. The cells were then challenged with seven concentrations of LPS from five species of bacteria with two strains from each bacterial species to make a total of 10 LPSs. The species and strains used were *Acinetobacter* genospecies 13 TU 4802 and 4809, *Haemophilus influenzae* 4838 and 4839, *Pseudomonas aeruginosa* 4789 and 4845, *Klebsiella pneumoniae* 4841 and 4844 and finally *E. coli* 4842 and 4843. The concentrations of LPS used were 100ng/ml, 10ng/ml, 1ng/ml, 100pg/ml, 10pg/ml, 1pg/ml and 0.1pg/ml. This procedure is described in full in chapter 2.4.3. The messenger RNA (mRNA) was extracted from the THP-1 cells that had been challenged with the different concentrations of LPS as described in chapter 2.5.3. The mRNA that had been extracted was turned into cDNA as described in chapter 2.5.4. The cDNA from *Acinetobacter* genospecies 13 TU 4809 was used as a guide to work out which two cDNAs, of the seven potential candidates, would give the best results. The cDNAs were run as described in chapter 2.5.6.2. The data from the QPCR experiment showed that for the 18S ribosomal RNA, TNF-α, IL-1β and IL-10 the cDNA from the cells challenged by 10ng/ml and 1ng/ml concentrations of LPS gave the best results, whilst for IFN-γ and IL-8, the cDNA from the cells challenged by 100ng/ml and 10ng/ml concentrations of LPS gave the best results.

3.3.4 Comparison of mRNA production between the bacterial species.

LPS from each strain of the species was used to measure the levels of production of mRNA from all the six genes of interest. The 18S ribosomal RNA was used as a positive control and also as the base line level of gene production in the THP-1 cells. The cycle threshold (Ct) value generated from the cDNA of the THP-1 cells challenged by the LPS from the different bacterial species and strains, for the 18S ribosomal RNA was used to normalise all the other genes' Ct values. The normalisation was carried out by dividing each ct value, from the cDNA generated by challenge with the LPS from the different bacteria, for each gene by the ct value of the 18S ribosomal RNA, cDNA from the same bacterial strain, to give a normalised Ct value. The results for the two *Acinetobacter* genospecies 13 TU strains tested are shown in Figure 3.31.

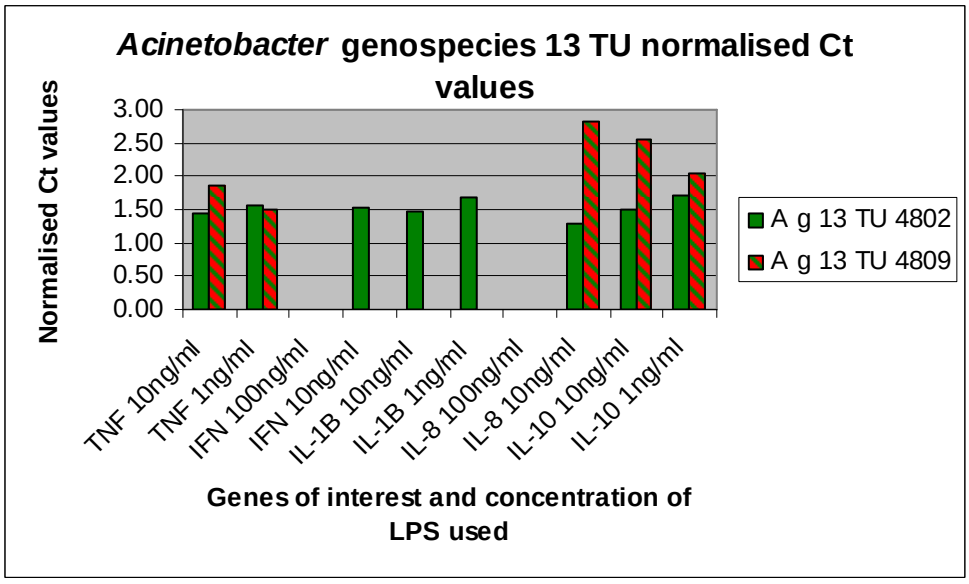

Figure 3.31: Levels of mRNA product from THP-1 cells for the genes of interest generated by challenge with LPS from *Acinetobacter* genospecies 13 TU 4802 and *Acinetobacter* genospecies 13 TU 4809. The results for both strains for each gene are plotted next to each other.

When analysing the results presented in Figure 3.31, it needs to be remembered that the smaller the bars the greater the amount of mRNA for that particular gene was extracted from the THP-1 cells. This is because the Ct value is the cycle at which the amount of RNA amplified has reached a threshold level and is now large enough to measure effectively. Thus the greater amount of mRNA in the sample, the faster this threshold is reached and so the Ct value is correspondingly lower. If the Ct value is zero though this means that the Ct value was not sufficiently greater than the negative control so the value is counted as zero since no detectable levels of cytokines were produced. The results were compared statistically through the use of 2-samples t-tests carried out in Minitab 15. Thus in Figure 3.31 it is possible to see that *Acinetobacter* genospecies 13 TU 4802 LPS generated a greater cytokine response than *Acinetobacter* genospecies 13 TU 4809 LPS. The difference between the levels of cytokine responses generated by the LPS of the two strains was not significant at $p<0.05$. This is probably due to the low number of data points involved in the experiments. *Acinetobacter* genospecies 13 TU 4809 LPS generated low level responses to IL-8 and IL-10 with lower responses to TNF-α than the *Acinetobacter* genospecies 13 TU 4802 LPS was able to generate. *Acinetobacter* genospecies 13 TU 4809 LPS was unable to induce a high enough cytokine response for IFN-γ and IL-1β, compared with the negative control for it to be considered anything but a zero level response. On the other hand *Acinetobacter* genospecies 13 TU 4802 LPS was able to generate a cytokine response to all the genes at a higher level than the LPS from *Acinetobacter* genospecies 13 TU 4809.

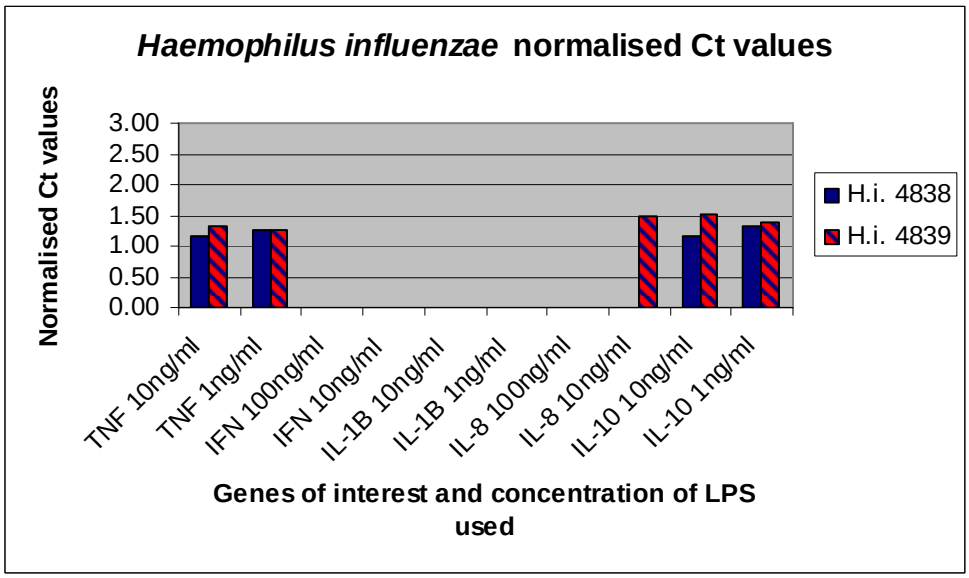

Figure 3.32: Levels of mRNA product from THP-1 cells for the genes of interest generated by challenge with LPS from *Haemophilus influenzae* 4838 and *Haemophilus influenzae* 4839. The results for both strains for each gene are plotted next to each other.

In Figure 3.32 it is possible to see that *Haemophilus influenzae* 4838 LPS generated a slightly higher, but not significantly so at $p<0.05$, cytokine response when compared with *Haemophilus influenzae* 4839 LPS. There was no significant production of mRNA for IFN-γ, IL-1β and IL-8 100ng/ml, when compared with the negative controls. There was a small amount of mRNA production in IL-8 10ng with *Haemophilus influenzae* 4839.

Although there is a difference between the two *Haemophilus influenzae* LPSs, it is not significant and it is less than the difference between the two *Acinetobacter* genospecies 13 TU LPSs in Figure 3.31. There was a significant difference $p<0.001$ between the cytokine production levels of the *Acinetobacter* genospecies 13 TU LPS and *Haemophilus influenzae* LPS. This is probably due to the fact that the *Haemophilus influenzae* LPS is unable to generate a response from either INF-γ or IL-1β and only LPS from *Haemophilus influenzae* 4839 was able to generate an IL-8

response. Thus whilst *Haemophilus influenzae* LPS seems to have generated higher levels of cytokine production of TNF-α and IL-10 than the Acinetobacter genospecies 13 TU LPS was able to, they were not able to generate production of the other cytokines as effectively as *Acinetobacter* genospecies 13 TU LPS.

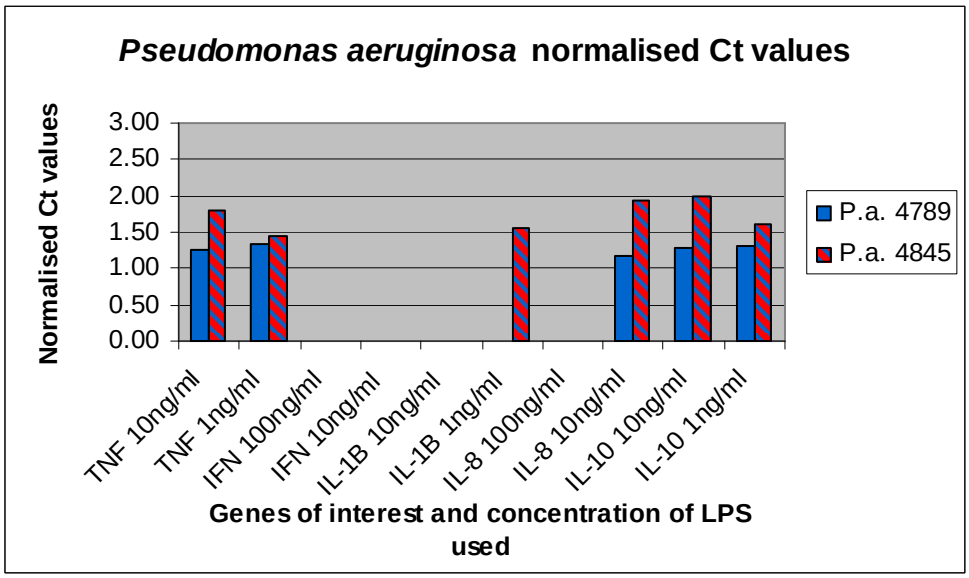

Figure 3.33: Levels of mRNA product from THP-1 cells for the genes of interest generated by challenge with LPS from *Pseudomonas aeruginosa* 4789 and *Pseudomonas aeruginosa* 4845. The results for both strains for each gene are plotted next to each other.

In Figure 3.33 it can be seen that *Pseudomonas aeruginosa* 4789 LPS generated a higher cytokine response than *Pseudomonas aeruginosa* 4845 LPS, but again this difference was not significant. The levels of IFN-γ, IL-1β 10ng/ml and IL-8 100ng/ml mRNA created in the cells at the time of RNA extraction, was not significantly different to those of the negative controls. There was a small amount of mRNA production for IL-1β from the cells challenged with 10ng of *Pseudomonas aeruginosa* 4845 LPS. LPS from both strains produced mRNA for IL-8 at 10ng/ml concentration of LPS, with *Pseudomonas aeruginosa* 4789 LPS inducing the highest levels.

There was no significant difference between the mRNA levels generated by *Pseudomonas aeruginosa* LPS and *Haemophilus influenzae* LPS. Whilst *Pseudomonas aeruginosa* LPS did not generate a significantly greater response than *Acinetobacter* genospecies 13 TU LPS, it can be seen that it generates greater cytokine levels than those generated by the *Pseudomonas aeruginosa* LPS.

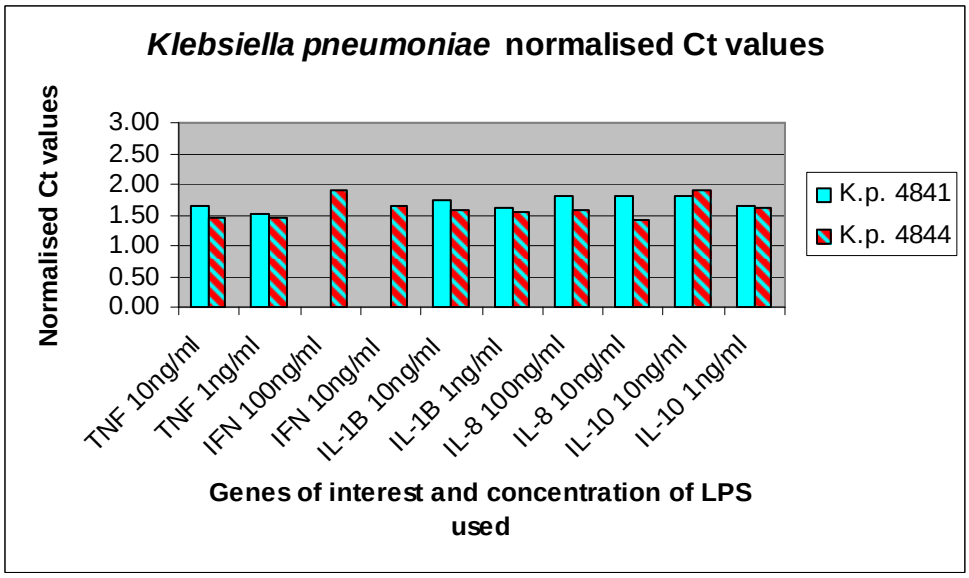

Figure 3.34: Levels of mRNA product from THP-1 cells for the genes of interest generated by challenge with LPS from *Klebsiella pneumoniae* 4841 and *Klebsiella pneumoniae* 4844. The results for both strains for each gene are plotted next to each other.

Figure 3.34 shows that *Klebsiella pneumoniae* 4844 LPS is able to induce an mRNA response to all the genes of interest. *Klebsiella pneumoniae* 4841 LPS was unable to induce mRNA production in IFN-γ. The levels of mRNA production between the LPS of the two strains were not significantly different, but *Klebsiella pneumoniae* 4844 LPS had very slightly higher levels of production.

The *Klebsiella pneumoniae* LPS induces mRNA levels at a lower levels than *Pseudomonas aeruginosa* and *Haemophilus influenzae* LPS for the cytokines that

were produced by all the strains. Due to the fact that *Klebsiella pneumoniae* LPS was able to generate cytokine production for all the cytokines, there was a significant difference $p<0.05$ between *Klebsiella pneumoniae* LPS and *Haemophilus influenzae* 4838 and *Pseudomonas aeruginosa* 4790 LPS. It also generated a greater mRNA production than *Acinetobacter* genospecies 13 TU LPS, especially *Acinetobacter* genospecies 13 TU 4809, but not significantly so.

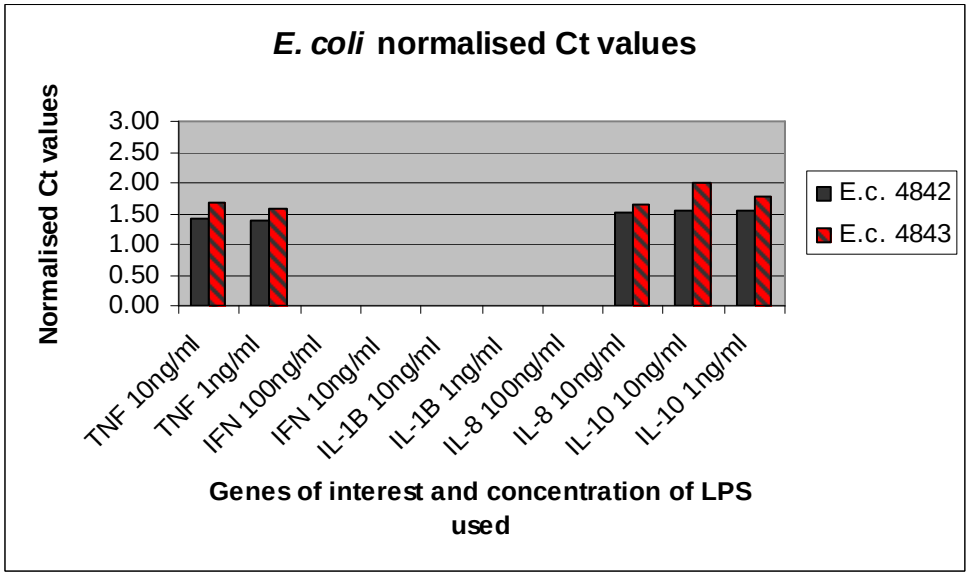

Figure 3.35: Levels of mRNA product from THP-1 cells for the genes of interest generated by challenge with LPS from *E. coli* 4842 and *E. coli* 4843. The results for both strains for each gene are plotted next to each other.

As Figure 3.35 shows, there was no significant difference between the LPS from *E. coli* 4842 and *E. coli* 4843; *E. coli* 4842 LPS generated a slightly higher level of cytokine production. Both strains of *E. coli* LPS were unable to induce mRNA production with IFN-γ, IL-1β and IL-8 100ng/ml.

The mRNA levels induced by *E. coli*, *Pseudomonas aeruginosa* and *Haemophilus influenzae* LPS were not significantly different. The *E. coli* LPS generates

significantly different $p<0.01$ levels of mRNA production with *Klebsiella pneumoniae* 4844 LPS. This is because *Klebsiella pneumoniae* 4844 LPS is able to induce cytokine production for all the cytokines, whilst the *E.coli* LPSs was only able to induce cytokine production in half the cytokines looked at in the study. When the level of cytokine production was compared for the cytokines that the *E. coli* LPS was able to induce, there was no significant difference between the LPSs from the two organisms. This is why there is no significant difference between *Klebsiella pneumoniae* 4841 LPS and the *E.coli* LPS. When compared with *Acinetobacter* genospecies 13 TU LPS there was no significant difference between these and the *E. coli* LPS, but it can be seen that *E. coli* LPS does induce higher cytokine levels especially when compared with those of *Acinetobacter* genospecies 13 TU 4809 LPS.

3.3.5 Comparison of mRNA production between genes of interest

The same results used to generate the previous five figures were used to generate a comparison between the levels of mRNA production of the different genes across the species.

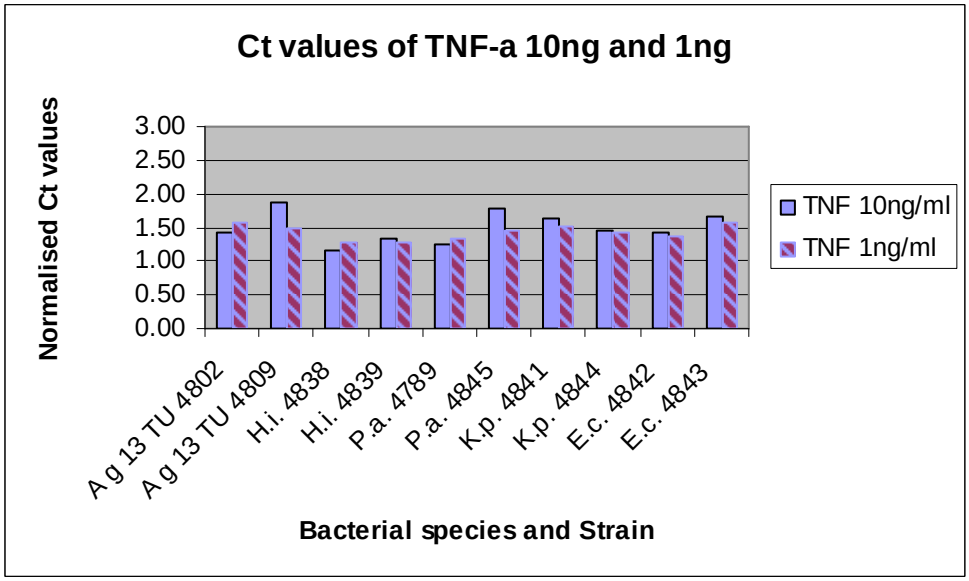

Figure 3.36: The normalised Ct values of TNF-α from cDNA taken from THP-1 cells challenged with either 10ng/ml or 1ng/ml of one of the ten different LPSs from the strains of bacteria.

Figure 3.36 shows that whilst there is some variation between the cytokine levels induced by the LPS of the strains, they are not significant. It can be seen that the two *Haemophilus influenzae* LPSs and the LPS from *Pseudomonas aeruginosa* 4789 are the highest inducers of TNF-α, whilst TNF-α mRNA level induced by LPS from *Acinetobacter* genospecies 13 TU 4802, *Pseudomonas aeruginosa* 4845, *Klebsiella pneumoniae* 4842 and *E. coli* 4843 was lower than the other LPSs. The final three LPSs again induced mid levels of mRNA for TNF-α production.

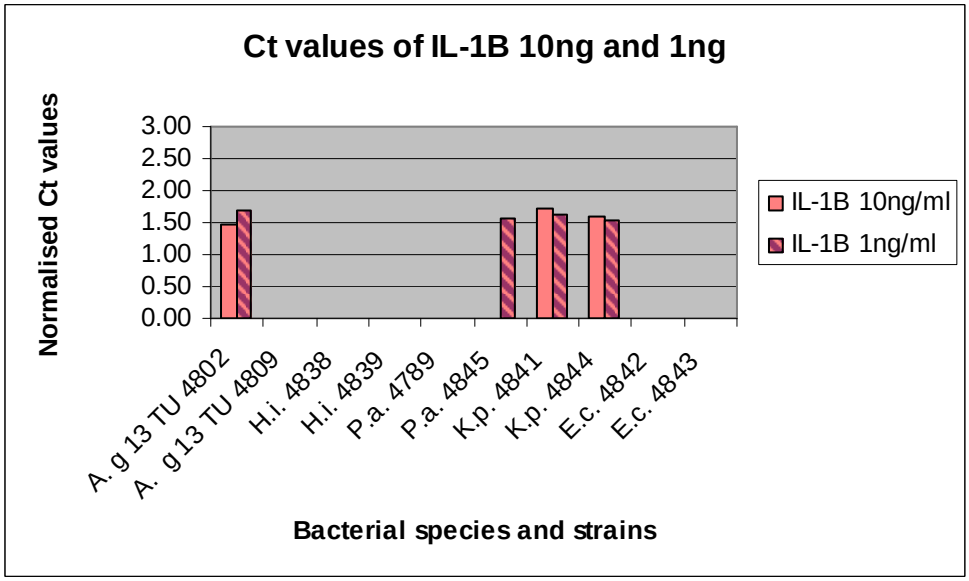

Figure 3.37: The normalised Ct values of IFN-γ from cDNA taken from THP-1 cells challenged with either 100ng/ml or 10ng/ml of one of the ten different LPSs from the strains of bacteria.

In Figure 3.37 it is possible to see that LPS from only a very small number of organisms were able to induce IFN-γ mRNA production. The sole exceptions to this were *Acinetobacter* genospecies 13 TU 4802 LPS,which induced the THP-1 cells to produce mRNA for IFN-γ when used at 10ng/ml, and *Klebsiella pneumoniae* 4844 LPS which was able to induce mRNA for INF-γ at both 100ng/ml and 10ng/ml. The two concentrations of LPS tested showed no significant difference between them.

The levels of mRNA isolated from the cells where IFN-γ mRNA was produced was significantly ($p<0.001$) lower than for the TNF-α mRNA.

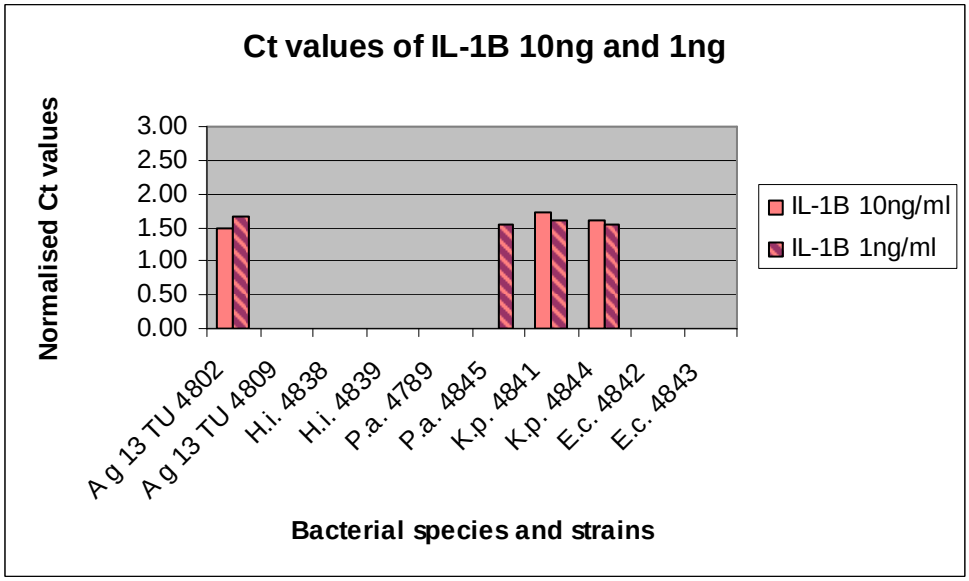

Figure 3.38: The normalised Ct values of IL-1β from cDNA taken from THP-1 cells challenged with either 10ng/ml or 1ng/ml of one of the ten different LPSs from the strains of bacteria.

In Figure 3.38 it can be seen that once again very few of the LPSs were able to induce mRNA production. The exceptions were again *Acinetobacter* genospecies 13 TU 4802 LPS, which was able to induce mRNA production for IL-1β at both 10ng/ml and 1ng/ml, and *Klebsiella pneumoniae* 4844 LPS which was also able to induce mRNA for IL-1β for both 10ng/ml and 1ng/ml. Moreover *Klebsiella pneumoniae* 4841 LPS was able to induce mRNA for IL-1β for both 10ng/ml and 1ng/ml, whilst *Pseudomonas aeruginosa* 4845 induced mRNA production at 1ng/ml. There was also no significant difference between the two concentrations of LPS used to stimulate the cells.

There was no significant difference between the cytokine production levels from IL-1β and IFN-γ, but there was a significant difference ($p<0.01$) between the cytokine levels produced for IL-1β and TNF-α.

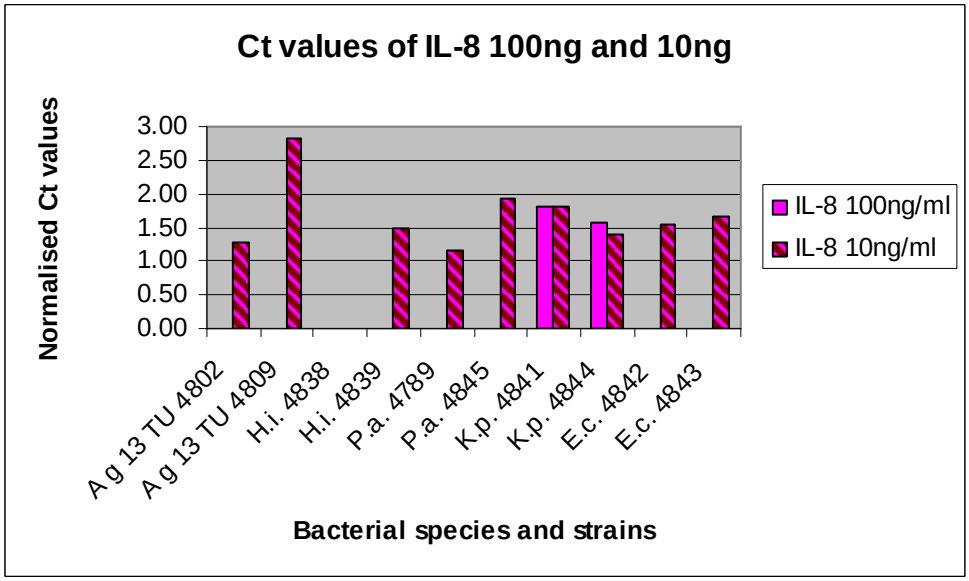

Figure 3.39: The normalised Ct values of IL-8 from cDNA taken from THP-1 cells challenged with either 100ng/ml or 10ng/ml of one of the ten different LPSs from the strains of bacteria.

Figure 3.39 shows that nearly all the LPSs were able to induce levels of mRNA production at 10ng/ml, but only *Klebsiella pneumoniae* 4841 and *Klebsiella pneumoniae* 4844 LPSs were able to generate mRNA production with 100ng/ml. There is a very wide variation in levels of mRNA production generated by the LPS from the different strains. *Acinetobacter* genospecies 13 TU 4802 and *Pseudomonas aeruginosa* 4789 LPSs had the highest mRNA responses and *Acinetobacter* genospecies 13 TU 4809 LPS had the lowest mRNA response. There is a significant difference ($p<0.01$) between the two concentrations of LPS used to stimulate the cells.

When compared with the other genes, it is possible to see that IL-8 at 10ng/ml was produced at significantly higher levels ($p<0.01$) than both IL-1β and IFN-γ. There was no significant difference between IL-8 at 10ng/ml and TNF-α production. For IL-8 at 100ng/ml though these results were inversed and there was no significant difference between it and IFN-γ and IL-1β. There was a significant difference ($p<0.01$) with TNF-α

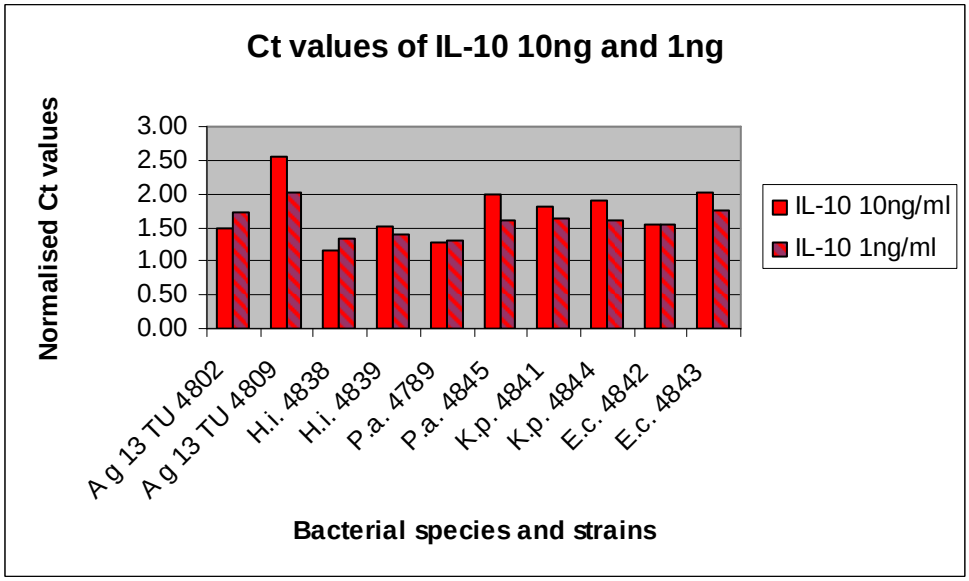

Figure 3.40: The normalised Ct values of IL-10 from cDNA taken from THP-1 cells challenged with either 10ng/ml or 1ng/ml of one of the ten different LPSs from the strains of bacteria.

In Figure 3.40 it can be seen that IL-10 was induced by all the LPSs at all dilutions. *Haemophilus influenzae* 4838 and *Pseudomonas aeruginosa* 4789 induced the highest levels of mRNA production, whilst easily the lowest level of mRNA production was *Acinetobacter* genospecies 13 TU 4809. The other LPSs fall in between these two extremes. There was no significant difference between the two concentrations of LPS.

When compared with the other genes, it can be seen that there is no significant difference between the levels of mRNA production for IL-10, TNF-α and IL-8 when stimulated with 10ng/ml of LPS. There was a significant difference ($p<0.05$) between the levels of production of mRNA for Il-10 and IFN-γ, there was also a significant difference ($p<0.05$) with IL-10 and IL-1β and IL-8 when the last two were stimulated by 100ng/ml of LPS.

4. Discussion

4.1 LPS structures

There are a wide variety of different types of LPS structures. As was covered in the Introduction there are traditionally three parts to the LPS: the O-polysaccharide chain, the core polysaccharide region consisting of the inner and outer cores and the Lipid A moiety. The structures of the LPSs of the species investigated in this Thesis need to be described.

E. coli traditionally has a smooth LPS i.e. with an O-polysaccharide chain. The two strains of *E. coli* investigated here both had a typical smooth LPS type with easily identifiable repeating units. The *Klebsiella pneumoniae* strains also followed the traditional types for their species, both having smooth LPS with large numbers of small repeating units, so they appeared as a continuous smear on the ammoniacal silver stain gel. The *Pseudomonas aeruginosa* LPSs seemed to have rough LPS strains i.e. where only the Lipid A moiety and the core polysaccharide are present. As to whether these are in fact rough LPS types is discussed in greater detail below (Erridge *et al,* 2002).

Haemophilus influenzae LPSs are often found in the rough or smooth-rough forms. A smooth-rough form is an LPS that has a shortened O-polysaccharide chain attached to the core polysaccharide. The LPS from the *Haemophilus influenzae* strain investigated in this study are all of the smooth-rough type. The *Acinetobacter* genospecies 13 TU strains investigated in the study appeared to be of the rough LPS

type, but this is unlikely to be the case as *Acinetobacter* genospecies 13 TU is normally a smooth LPS. This is considered in Part 4.2 of the Discussion.

4.2 The LPS extractions.

As was seen in the ammoniacal silver stained PAGE gels and the colloidal gold stains, the re-extraction of LPS removed the residual protein from the LPSs. When the ammoniacal silver stain was carried out on *Acinetobacter* genospecies 13 TU LPS, these appeared to be rough LPS, as there was no banding pattern seen. This seemed strange, as so far all the published structures of *Acinetobacter* genospecies 13 TU LPS have been smooth LPS (Haseley *et al*, 1997a; Haseley *et al*, 1997b; Haseley *et al*, 1997c; Haseley *et al*, 1998; Pantophlet *et al*, 2001; Vinogradov *et al*, 2002; Vinogradov *et al*, 2003; MacLean *et al* 2009). The reason that this occurred, is most likely due to the fact that the sugar composition of the O-polysaccharide chain is lacking in periodic-hydroxyls.This means that the oxidising agent, periodic acid, is unable to cleave to any of the sugars of the O-chain and open up sites in the repeating unit to the ammoniacal silver, which is then unable to bind to the O-polysaccharide repeating units (Kropinski *et al*, 1985; cited by Kropinski *et al*, 1986). Thus when the developer is added to the gel there is no ammoniacal silver in the O-polysaccharide for it to react with. This is why there is no banding pattern with *Acinetobacter* genospecies 13 TU LPS on the PAGE gels stained with ammoniacal silver stain. The two *Pseudomonas aeruginosa* strains are also possibly smooth LPS that appear to be rough LPS, as they also sometimes lack periodic-hydroxyls in their O-polysaccharides (Kropinski *et al*, 1985; cited by Kropinski *et al*, 1986), but there are also many rough LPS strains of *Pseudomonas aeruginosa*. The other three species LPSs were perfectly normal for their species types.

4.3 Limulus Amoebocyte Lysate (LAL) assay

The results of this experiment showed that the endotoxic activities of the majority of the LPSs extracted were at very similar levels, falling between 100 endotoxicity units/μg of LPS and 300 endotoxicity units/μg of LPS. For these units to be best understood, it must be remembered that 1 endotoxicity unit is equal to 0.2ng of a standard *E. coli* LPS. However, several of the LPSs were below the 100 endotoxicity unit/μg of LPS. These were largely from *Acinetobacter* genospecies 13 TU with four of the seven strains coming from this species. The other species were *Haemophilus influenzae*; *Pseudomonas aeruginosa* and *E. coli* with one strain each falling below 100 endotoxicity units/μg of LPS. Two strains though had much higher levels of endotoxicity than the rest, namely *Acinetobacter* genospecies 13 TU 4802 and *Klebsiella pneumoniae* 4841, which had 600 and 1000 endotoxicity units/μg of LPS respectively. This was more than twice as large as the next highest endotoxicity level. The endotoxicity units are an indication of how "aggressive" the LPS is, so the higher the endotoxicity units are, the more potent they are at stimulating the immune system. Thus it can be said that the LPSs from *Acinetobacter* genospecies 13 TU 4802 and *Klebsiella pneumoniae* 4841 seem to be the most potent stimulators of the immune system of these LPSs extracted.

Half of the LPSs isolated from strains of *Acinetobacter* genospecies 13 TU were at a very low endotoxicity level and so will not stimulate the immune system very strongly. These were very close in levels to those of LPSs extracted from one of the strains of *Pseudomonas aeruginosa*, one of the *Haemophilus influenzae* strains and one of the strains of *E. coli*. This is as expected, since *Acinetobacter* genospecies 13 TU is more of an opportunistic pathogen than a truly pathogenic species. The low

endotoxicity level of *E. coli* and *Haemophilus influenzae* LPSs is possibly due to the fact that they might be from strains that are less pathogenic as well or have other virulence factors, such as toxin etc. It is also possible that the some of the results are incorrect due to the poor solubility of LPS in water. LPS when dissolved in water forms clumps which means that when some of the mixture is removed the amount of LPS within is not indicative of the true amount of LPS in the solution. This is because it is possible to obtain either large amounts of LPS in clumps or nearly no LPS if a clump is not drawn up. The way this problem was attempted to be limited was to dissolve the LPS in 0.2% Triethylamine (TEA). This molecule helps the LPS dissolve and stops the formation of clumps, thus allowing the LPS to diffuse fully through the liquid.

As is discussed above, *Acinetobacter* genospecies 13 TU 4802 has a very high endotoxicity level possibly meaning that it is from a strain more suited to actively infecting people. The other three LPSs from *Acinetobacter* genospecies 13 TU were at similar levels of endotoxicity to LPS from three of the *Haemophilus influenzae* strains and close to those of half of the LPSs from *Klebsiella pneumoniae* and *E. coli* strains. This indicates that these strains are possibly entering niches closer to those occupied by these more specialised human commensals. These three LPSs of *Acinetobacter* genospecies 13 TU are also very close in level to that of *Pseudomonas aeruginosa* 4789 LPS, although slightly higher than it. *Pseudomonas aeruginosa* LPS is often thought to be less endotoxic than many other pathogenic Gram-negative organisms (Goldberg & Pier, 1996; Erridge *et al*, 2007). As was shown by Erridge *et al* (2007) *Acinetobacter* genospecies 13 TU LPS is a potent stimulator of the immune system and is able to stimulate TNF-α and IL-8 production at the same level as LPS from *E. coli* signalling through TLR-4. Thus whilst the endotoxicity levels from the LAL

assay vary between very high levels and other much lower levels, the LPS has always stimulated a response.

The LPS from the *Haemophilus influenzae* 4789, 4838 and 4839 strains is mostly at the middle level lying between 100 and 300 endotoxicity units/μg of LPS, with LPS from 4838 and 4839 being closer to 300 endotoxicity units/μg of LPS and the LPS from 4789 being closer to 100 endotoxicity units/μg of LPS. *Haemophilus influenzae* 4840 had very low levels of LPS endotoxicity. So the LPSs from the three higher strains are more endotoxic than the LPS belonging to the low level strain. It is possible that the low endotoxicity strain is less pathogenic than the other three, although it is likely to have some pathogenic potential, as it and all the other strains tested here were isolated from patients with respiratory tract infections.

4.4 Range of antibodies to *Acinetobacter* genospecies 13 TU LPS in a healthy population of Southeast Scotland

4.4.1 Inter strain comparisons

Acinetobacter genospecies 13 TU is now a fairly common hospital acquired bacterium and infections caused by it have been on the rise over the last twenty years (Giamarellou *et al*, 2008). There has never been a study to attempt to see how widespread exposure to this organism is in the wider healthy population. To attempt to ascertain this, it was decided to investigate the levels of antibody to LPS from eight *Acinetobacter* genospecies 13 TU strains isolated from patients in the Royal Infirmary Edinburgh. The measurement of the antibody levels to *Acinetobacter* genospecies 13 TU LPS might serve as an indicator of how widespread exposure to *Acinetobacter* genospecies 13 TU is. It does need to be noted that very little is known about the

differences between the different structures of LPS within the genus *Acinetobacter*. So it is quite possible that antibody levels to *Acinetobacter* genospecies 13 TU LPS are in fact due to exposure to a different species of *Acinetobacter*. Since the higher the antibody levels, the greater is the number of people that will have been exposed to the LPS. Since exposure to the LPS normally occurs with exposure to the bacterium as well. So LPS exposure can be used as a rough estimate on the levels of exposure to the bacteria. The LPSs were used to assess the antibody levels in serum samples from 475 blood donors by ELISAs. Blood donors are likely to represent the healthy population, since one must be in good health to be allowed to give blood. It was decided to use 475 blood donors since this was considered a large enough number to act as a reasonable indicator of the wider healthy population as a whole.

There are some issues with the above assumption, in that there is not a direct correlation between the antibody levels to LPS and exposure to the organism itself. Since some LPSs appear to generate a much greater antibody response than others. This means that if 50% of the population has been exposed to the potent LPS then each individual that has been exposed to it will have a higher response and so the mean of the overall response would be much higher, than the mean antibody response to an LPS from a different species or strain that is much less potent, but with an exposure rate of 70%. In the above case when the antibody levels are observed, it will appear that the strain with a 50% exposure will have a much higher mean and seem to have a much higher exposure rate than the strain with a 70% exposure rate. Alongside this issue is the problem of cross-reactivity, which is where the antigen closely resembles another antigen and so the antibodies will be able to bind to both of them. So in this case this would mean that an LPS to a more common strain has some similarities in binding sites to the stain of investigation. This can artificially increase

the apparent antibody levels to the specific LPS, as there are now not only whatever antibodies that would normally be present in the serum against the specific LPS, but also any antibodies that would bind to the other LPSs as well. To overcome these problems it was necessary to observe all the data in one go and not just take the mean as the only important factor. Also inhibition ELISAs were carried out to measure the rate of cross-reactivity between the strains. Unfortunately the inhibition ELISAs did not work effectively and so were unable to be completed. This will be covered in a later part of the Discussion.

The results showed that there was a definite variation in antibody levels to the different LPSs from the eight *Acinetobacter* genospecies 13 TU strains. This suggests that the different strains have different exposure rates within the healthy population of Southeast Scotland. LPS from *Acinetobacter* genospecies 13 TU strains 4793, 4799 and 4801 generated the highest mean levels of antibody, but also had the widest base of antibody results with a good mixture of results, making it likely that the higher mean is in fact due to a wider exposure to the LPS of the organism. The LPS from *Acinetobacter* genospecies 13 TU strains 4800 and 4809 generated antibody responses whose means are very close to each other and also have a wide range of antibody responses. This indicates that the exposure to the two *Acinetobacter* genospecies 13 TU strains is not a bi-modal distribution, which would indicate a smaller exposure, but a higher antibody response from those that were exposed. The means are lower than those to the LPS of the previous three strains, which would indicate that the exposure to *Acinetobacter* genospecies 13 TU 4800 and 4809 is lower than the previous strains. The LPS from *Acinetobacter* genospecies 13 TU 4808 generates a lower mean antibody response than the previous five strains. Once again though there is no bi-modality so the mean can be read as an accurate exposure rate over the

population. Again this strain has less exposure within the population than the previous *Acinetobacter* genospecies 13 TU strains. The LPS from the final two *Acinetobacter* genospecies 13 TU strains 4802 and 4803 have the lowest means of all and have very high numbers of antibody results between 1.5-3.5 OD 405nm; these are the lowest levels of antibody response to the LPSs out of all the *Acinetobacter* genospecies 13 TU strains. The results indicate that these two strains have the lowest exposure in the healthy population of Southeast Scotland. So as can be seen there is a definite variation in the exposure rate to the different strains, with some strains having a higher rate of exposure whilst some having a low rate of exposure. In fact, the strain of *Acinetobacter* genospecies 13 TU with the highest endotoxic activity is also the strain with the lowest exposure rate of its LPS to the healthy population, possibly indicating that it is a more specialised pathogenic strain, which is more commonly found in hospitals and not in the healthy population.

4.4.2 Inter species comparisons

The antibody levels to the LPS from the *Acinetobacter* genospecies 13 TU strains were also compared with antibody levels generated by LPS from three organisms that commonly cause respiratory tract infections (Dupont *et al*, 2003). These organisms were *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli*, and two strains of each were tested. Each serum sample was averaged across the eight *Acinetobacter* genospecies 13 TU, the two *Pseudomonas aeruginosa*, the two *Klebsiella pneumoniae* and the two *E. coli* strains and the mean antibody levels to the LPSs from these different species were compared.

The comparison of the mean antibody levels to *Pseudomonas aeruginosa* and *Acinetobacter* genospecies 13 TU showed that there was a significant difference

between the two means. But even with this significant difference the means were much closer to each other than they were for the antibody levels to the LPS *Acinetobacter* genospecies 13 TU and *E. coli*. The points also overlay each other fairly well thus also showing a similarity between the two sets of data. *Acinetobacter* genospecies 13 TU is a human pathogen, unlike *Pseudomonas aeruginosa*, but as is discussed below a closely related species to this organism is a common environmental organism. Otherwise if that is not what the organism is responding to, then a common nosocomial infection will have a lower exposure in a healthy population. This is because they will not have been exposed to it as regularly, since it isn't really found outside of hospitals.

As covered in the Introduction there is currently some discussion as to whether *Acinetobacter baumannii* is in fact an environmental organism or indeed as common in hospitals as it appears to be. So the natural reservoir for the organisms has never been discovered (Van looveren *et al*, 2004; Perez *et al*, 2007; Peleg *et al*, 2008). The majority of the literature though asserts that *Acinetobacter baumannii* is an environmental organism (Paterson, 2006; Giamallerou *et al*, 2008). The probable reason that is put forward as to why it is possible for the majority of the papers to claim the *Acinetobacter baumannii* is an environmental organism, is that until recently *Acinetobacter baumannii* was not easily differentiable from other closely related *Acinetobacter* spp. by normal phenotypic methods. The development of 16s-23S DNA/DNA hybridisation by Tjernborg & Ursing in 1989 allowed the differentiation between the different species, which allowed for a more complete phylogenetic picture of *Acinetobacter* spp. to be developed. There are now 32 genomic species within the group (Van Looveren *et al*, 2004). *Acinetobacter baumannii* and *Acinetobacter calcoaceticus* and two other genospecies 3 and 13 TU

make up a very closely related group of species often referred to as the *Acinetobacter calcoaceticus* - *Acinetobacter baumannii* complex. These species are almost impossible to differentiate from each other by normal phenotypic methods and thus it is likely that a number of the species previously described as *Acinetobacter baumannii* may in fact have been *Acinetobacter calcoaceticus* isolates that were misidentified as *Acinetobacter baumannii*. *Acinetobacter calcoaceticus* is in fact a common environmental organism that is found ubiquitously throughout nature (Peleg *et al*, 2008). So the argument put forward is that the majority of strains that have been previously identified in nature as being *Acinetobacter baumannii* are in fact *Acinetobacter calcoaceticus*. So now it has entered the general consciousness that *Acinetobacter baumannii* is an environmental organism all papers state this without checking to whether this is true. This has led to the probable mis-representation of *Acinetobacter baumannii* as an environmental organism. Whilst this means that *Acinetobacter baumannii* is not as commonly isolated as thought, the organisms are extremely closely related so it is likely that the data isolated for the *Acinetobacter* genospecies 13 TU can be also be applied to *Acinetobacter baumannii*.

Thus it has been established that there are great similarities between these species of *Acinetobacter*, which means that it is probably likely that they share the same basic core and lipid A structure of the LPS. This obviously would mean that the antibody levels we are detecting to the *Acinetobacter* genospecies 13 TU LPS could in fact be antibodies developed against the core of *Acinetobacter calcoaceticus* LPS. This could mean that the actual level of antibody to *Acinetobacter* genospecies 13 TU LPS is lower than these results would suggest.

The comparison between the means of the antibody levels against LPS from *Klebsiella pneumoniae* and *Acinetobacter* genospecies 13 TU showed that there was a significant difference between the means. The difference was slightly higher than the difference between the means of the antibody levels against LPS from *Pseudomonas aeruginosa* and *Acinetobacter* genospecies 13 TU. The means of the antibody levels against LPS from *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were not significantly different. This is not what would be expected normally, as *Pseudomonas aeruginosa* is a common environmental organism, whilst *Klebsiella pneumoniae* is a common commensal organism of the gut. So it is odd to have the mean antibody results against the LPS from the organism being so close, as it would be expected for *Klebsiella pneumoniae* to have a much higher level of exposure in the healthy population. When the individual points on the graph are compared, it is possible to see that whilst *Klebsiella pneumoniae* has much greater number of higher responses it also has a much greater number of lower responses. This could be taken as an indication that *Klebsiella pneumoniae* has been exposed to a smaller part of the of the population, but has generated a higher response in several individuals that were exposed to the organism, thus giving a mean that is higher than that against the LPS of *Acinetobacter* genospecies 13 TU. This is one possibility, but another is that, when the two strains that were averaged to make the results for *Klebsiella pneumoniae*, are looked at, then it is easy to see that they are significantly different ($p<0.01$) from each other, with *Klebsiella pneumoniae* 4841 having a much higher mean than that of *Klebsiella pneumoniae* 4844. The reason for this discrepancy between the two strains is possibly due to one of the strains being a more specialised pathogenic strain as opposed to a more common gut commensal. The more specialised pathogen would be more common in hospitalised patients and so be less likely to have exposure in the

healthy population. Exposure to the common gut commensal would be much more widespread as it will be commonly found in the gut of the population. The mean of the higher antibody response of 0.623 is much closer to the mean of *E. coli* at 0.689 than it is to the mean of *Klebsiella pneumoniae* 4844 of 0.538, which is definitely closer to that of *Acinetobacter* genospecies 13 TU which is 0.471. Another possibility is that with only two strains of *Klebsiella pneumoniae* LPS being used, it means that the antibody level to them will be a combination of anti-core and anti-O chain, specific for each particular LPS antibody. This could lead to the discrepancy between the LPS of the two strains. There was a greater number of *Acinetobacter* genospecies 13 TU LPSs tested which will be likely to reduce the importance of the specific antibodies, as the anti-core antibodies are enhanced, since there will be more of them across the strains. To try and see what the actual reason is for this discrepancy, it would be best to obtain a number of extra *Klebsiella pneumoniae* strains and measure the antibody response to their LPSs. These responses could then be used to see if this is a common feature for *Klebsiella pneumoniae* or is something that just affects the two strains isolated here.

When the means of the antibody responses to LPS from *E. coli* and *Acinetobacter* genospecies 13 TU were compared, it was possible to see that there was a significant difference between the two means. When the scatterplot was also investigated it could also be seen that the *E. coli* points were much more often above the points from *Acinetobacter* genospecies 13 TU. As with *Klebsiella pneumoniae* strains above there were only two strains of *E. coli* used in the experiment. So as above there could be a great discrepancy between the more general core antibodies and the specific O-chain antibodies. Also *E. coli* has 5 core types and with only two samples it is impossible for them all to have been represented in the experiments carried out. Again an

increased number of organisms could have helped even out the results. However, they would probably still have been similar to the results currently here. This is as was expected, since *Acinetobacter* genospecies 13 TU is, as has been discussed above, a nosocomial infection, whilst *E. coli* is the most common facultative anaerobic gut organism in the majority of the population.

So it can be said that the antibody levels to *Acinetobacter* genospecies 13 TU LPS are at a similar level to those of *Pseudomonas aeruginosa* LPS. It is at a much lower level of exposure when compared to *E. coli* LPS, but again this is as expected. With *Klebsiella pneumoniae* it was odd as it is also a common gut organism and the antibody levels were close, but there are a variety of reasons for this as discussed above. Overall then it is possible to say that exposure to *Acinetobacter* genospecies 13 TU is about as widespread in the healthy population of Southeast Scotland as *Pseudomonas aeruginosa* and at least one strain of *Klebsiella pneumoniae.*

4.5 Inhibition assays

These experiments were carried out to attempt to ascertain the level of cross-reactivity between the strains. This was carried out firstly with three different *E. coli* LPSs to test the theory. This experiment was successful. When the same method was attempted using *Acinetobacter* genospecies 13 TU LPSs there was no inhibition at all; in fact even the strain that was bound to the plate seemed to have no inhibitory action against itself. A reason as to why this may have happened is that in solution the *Acinetobacter* genospecies 13 TU LPS changes confirmation in such a way that it deforms the binding sites where the antibodies would normally bind to in the normal confirmation of the LPS. Possible ways to test this theory would be to obtain

microbeads and bind the lipid A moiety to these. These LPS coated microbeads could then be incubated with the serum as the solubilised LPS was in the original experiment. The ELISA would then be carried out as normal. Another option would be using whole heated killed bacteria, but this has other risks involved, such as a much greater chance of cross reactivity between the LPS and other surface antigens and so the experiment would be less certain in its conclusions.

4.6 Range of antibodies to *Haemophilus influenzae* LPS in a healthy population of Southeast Scotland

4.6.1 Inter strain comparisons

The antibody levels to the LPSs of three of the *Haemophilus influenzae* strains 4838, 4839, and 4840, were very similar with the ranges of their antibody levels having a very similar mean and standard deviation. This indicates that either their LPSs are similarly widespread within the healthy population of Southeast Scotland or that there is a common determinant (the core) across the LPS of the strains that they are all responding to. The antibody levels to the LPS from *Haemophilus influenzae* 4790 on the other hand have a much greater mean and greater standard deviation. This is due to the extra group of donors with an OD at 405nm of 1.7-1.8, to the LPS of *Haemophilus influenzae* 4790. This extra peak seems to indicate a number of individuals who have developed a much greater amount of antibody to this LPS. The reason for this could be that these individuals have been exposed to a higher level of the LPS than other people indicating a more recent and greater exposure to the bacterial strain itself. Alternatively this particular LPS induces a stronger antibody

response from the immune system of certain individuals, which is why they have high levels of antibody to it.

The shape of the curve Figure 3.20 suggests a certain level of bimodality to the antibody levels generated by the LPS to *Haemophilus influenzae* 4790. This is where the normal distribution is not strictly accurate as the graph has two peaks rather than just the one that a normally distributed graph would have. This means that a normal distribution will not work on this type of graph. In Figure 3.20 though this is likely to be the case as the second peak is much smaller than the first peak and it is more likely that this occurred because of a difference in which region of the LPS that the antibodies bound to than a true bimodal population. Different parts of the LPS can induce a greater antibody response than others and if some of a population responded to one of these areas they would have a much greater level of response than other individuals who were responding to another part of the LPS that induced a lower response. Even with this second peak, the results of the antibody levels to the LPS from *Haemophilus influenzae* 4790 are consistently higher than those of the other three strains. So it seems that *Haemophilus influenzae* 4790 is the strain which has the most widespread exposure in the healthy population of Southeast Scotland.

4.6.2 Inter species comparisons

The antibody levels to the LPSs from all the *Haemophilus influenzae* strains were analyzed in the same manner as with the antibody levels to all the *Acinetobacter* genospecies 13 TU strains. The antibody levels to *Haemophilus influenzae* LPS when averaged across all four strains were compared with the antibody levels to the LPS from the same three reference species as before, namely *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli*.

When the antibody levels to LPS isolated from *Pseudomonas aeruginosa* and *Haemophilus influenzae* are compared, it can be seen that there is a significant difference between them, with *Haemophilus influenzae* having by far the greater antibody level. This is as expected, since *Haemophilus influenzae* is a very common commensal respiratory tract organism (World Health Organisation, 2005) and *Pseudomonas aeruginosa* is a common environmental organism. So it is expected for the common commensal to have a higher presence in the healthy population than an environmental organism.

The comparison between the antibody levels to the LPS from *Klebsiella pneumoniae* and *Haemophilus influenzae*, showed that there was a significant difference between the antibody levels, with antibody levels to *Haemophilus influenzae* LPS being much greater than those of *Klebsiella pneumoniae*. Although both are commensals as was affirmed in Part 4.3.2 of the Discussion the two strains of *Klebsiella pneumoniae* are hugely varied, thus it is hard to draw definite conclusions from these two results. Even when looking at the means of antibody levels to the LPS from the two *Klebsiella pneumoniae* strains, they were both significantly lower than the antibody levels of LPS from *Haemophilus influenzae*. Currently then it seems that exposure to *Haemophilus influenzae* is much more widespread than *Klebsiella pneumoniae* in the healthy population of Southeast Scotland.

When the antibody levels to the LPS from *E. coli* and *Haemophilus influenzae* were compared, they showed that there was a significant difference between the antibody levels. However, the difference between their means was much smaller than it was between the other two species. Thus it seems that exposure to these two organisms is

roughly the same. This is also as expected, since both are common commensal organisms.

Thus it is possible to say that exposure to *Haemophilus influenzae* is slightly more widespread than that of *E. coli* in the healthy population of Southeast Scotland. It is more widespread than either *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*.

4.7 High responders

As was seen in Figure 3.28 there are some individuals, who have high antibody responses to all LPS antigens they are exposed to. These high responders could possibly be used to act as donors for passive vaccinations for people in high risk situations. These could include people who are about to undergo surgery or people in the ICU on ventilator machines. There has been some thought of using anti-endotoxin antibodies to help treat people suffering from Gram-negative infections (Ziegler *et al*, 1982; Baumgartner *et al*, 1985; Teng *et al*, 1985; Kirkland *et al*, 1986; Charalambous *et al*, 2007). The antibodies from these high responders, if used very carefully, could be of great benefit, with the massive increases in antibiotic resistance, as it much more unlikely that resistance will be developed to these. On the other hand, it is possible for antibodies to "accidentally" have cross-reactivity with common antigens from the patient being treated and start an auto-immune reaction. There is also the risk of blood borne viruses, such as HIV or Hepatitis B+C being spread by contaminated blood. This is why any use of this vaccine would have to be carefully monitored and all the blood fully screened for infections before it could be used.

4.8 Cytotoxin production from THP-1 cells with LPS

There was a great deal of variation between the different levels of cytokines induced by the different LPSs. Some cytokines were barely produced at all, whilst other cytokines were produced at higher levels. The cytokine which had the highest level of production was TNF-α. This cytokine was induced by the LPSs of all the bacterial strains studied. When the different levels of TNF-α productions were compared across the species and strains there was no significant difference between them; but with only ten data points in each group it is highly unlikely that this analysis is truly significant; for this to be so there would need to be a greater number of points. The reason statistics were used to analyse the results though was just in case they showed that there was a significant difference between the species or genes. However, when viewed "by eye" it is possible to see that *Haemophilus influenzae* had the highest level of production of TNF-α, with one of the *Pseudomonas aeruginosa* strains. TNF-α being the highest produced cytokine was not particularly surprising as the LPS had been incubated with the cells for 4 hours, which is enough time for TNF-α to have started being produced, whilst a number of other cytokines have not really started to be produced yet (Agarwal *et al*, 1995). This is because TNF-α is a potent pro-inflammatory cytokine, which is normally the first produced by the immune system when it comes into contact with an invading organism.

For IFN-γ there were very low levels of production. Only *Acinetobacter* genospecies 13 TU 4802 and *Klebsiella pneumoniae* 4844 actually induced any and even then it was produced at significantly lower levels than were produced for TNF-α. This is again as expected, since IFN-γ only normally starts to be produced after 10-18 hours, so low levels of production are perfectly normal (Chan *et al* 1991; Janskỳ *et al*, 2003).

IL-1β also had very low levels of production with only four strains producing it, namely the two *Klebsiella pneumoniae* strains, *Pseudomonas aeruginosa* 4845 and *Acinetobacter* genospecies 13 TU 4802. Again the production levels were significantly below the production of the TNF-α. This result was not expected as IL-1β is normally produced at the same time as TNF-α (Baron *et al*, 1993; Agarwal *et al* 1995). This could be due to these LPSs not being able to stimulate the cells for IL-1β production.

IL-8 though has a significantly larger amount being produced than either IL-1β or IFN-γ and there is no significant difference with TNF-α. It is induced by all strains except *Haemophilus influenzae* 4838. This is what is expected as well, since it is also a potent pro-inflammatory cytokine that is often stimulated within four hours (Baron *et al*, 1993; Agarwal *et al* 1995).

IL-10 is an anti-inflammatory cytokine that acts as a control mechanism on pro-inflammatory cytokines such as TNF-α and IL-8. It is often produced alongside the pro-inflammatory cytokines to control the immune response and make sure it doesn't become too potent and start to cause damage to the host (D'Andrea *et al*, 1993). There was no significant difference between IL-10, IL-8 and TNF-α, but it could be seen that IL-10 was produced at a slightly lower level than either IL-8 or TNF-α, but not significantly so. All species and strains induced this cytokine. It has been shown by Janskỳ *et al*, (2003) that there is a peak of IL-10 production after 4 hours followed by a reduction in production until 12 hours have passed, when there is a much larger production of IL-10. The initial peak is probably to reduce the initial immune response to make sure it does not increase too rapidly; the second peak is what keeps

the main immune response under control and shuts it down before any histological damage can be caused.

Haemophilus influenzae LPS induced the highest levels of production of the cytokines, in the cytokines that it could induce i.e. TNF-α and IL-10. IL-8 was also able to be induced at high levels by *Haemophilus influenzae* 4839 LPS. When compared with *Klebsiella pneumoniae* LPS which was able to induce production of all the cytokines, there was a significant difference between them. *Acinetobacter* genospecies 13 TU 4802 LPS was able to induce production of all the cytokines, whereas *Acinetobacter* genospecies 13 TU 4809 LPS was unable to induce production of either IFN-γ or IL-1β and the ones it could induce were at lower levels when compared with *Acinetobacter* genospecies 13 TU 4802 LPS. *Pseudomonas aeruginosa* and *E. coli* LPSs did not have significantly different production levels to each other or to *Haemophilus influenzae* LPS as they were able to induce mostly the same cytokines. *Pseudomonas aeruginosa* LPS was also able to induce production of IL-1β at low levels.

From the data seen here and when compared with the endotoxicity levels calculated using the LAL assays, it would be assumed that *Acinetobacter* genospecies 13 TU 4802 and *Klebsiella pneumoniae* 4841 would be the two most potent stimulators of the immune system and thus stimulate greater production of cytokines. This does not seem to be the case as there is no significant difference between the abilities of the LPS from the same strains to stimulate different production rates of the cytokines also whilst *Acinetobacter* genospecies 13 TU 4802 LPS induced higher production levels of cytokines than *Acinetobacter* genospecies 13 TU 4809 LPS. *Klebsiella pneumoniae* 4841 LPS in fact induced less cytokine production than *Klebsiella pneumoniae* 4844

LPS , but not significantly so, despite *Klebsiella pneumoniae* 4841 LPS having five times the endotoxicity levels by LAL. Thus it seems that endotoxicity levels calculated by the LAL assay were not indicative of how potent a cytokine stimulator a particular LPS is.

There was only one time point used in this experiment due to problems growing the cells which led to a long wait for viable cells to be grown to sufficient numbers to be able to experiment on them properly. With more time for the project, extra time points at 12 hours, 18 hours, 24 hours and 48 hours for all the LPS samples would have been included to allow for better understanding of the production of the cytokines that had not started to be produced after 4 hours.

4.9 Conclusions

So in conclusion it can be said that exposure to *Acinetobacter* and *Haemophilus influenzae* LPSs in a healthy population varies within the species. This is due to the fact that some strains with in the species had higher levels of antibody to the LPS in the healthy population than others. Apparent exposure to *Acinetobacter* genospecies 13 TU LPS was at a similar level of exposure to *Pseudomonas aeruginosa* LPS and also to at least one LPS from one of the studied strains of *Klebsiella pneumoniae* in a healthy population. *Haemophilus influenzae* LPS on the other hand had a similar level of exposure to *E. coli* LPS in a healthy population. This is useful to know as it has previously been unknown how widespread exposure to *Acinetobacter* LPS is outside a hospital setting. The level of exposure to *Acinetobacter* LPS is similar to that of *Pseudomonas aeruginosa* LPS, which suggests that it is not a commensal organism and could possibly be an environmental organism.

TNF-α is the most produced cytokine along with IL-8 and IL-10 close behind it in production levels. IFN-γ and IL-1β were at a lower level, but are not normally activated till 18 hours or so after the initial contact with the foreign antigen. *Haemophilus influenzae* LPS did not induce production of many of the cytokines, whilst *Klebsiella pneumoniae* LPS was able to induce production in all the cytokines investigated. *Acinetobacter* genospecies 13 TU LPS varied between the two strains with one being a potent inducer of cytokine production, whilst the other was not a very potent inducer. It was also seen that endotoxic activity is not necessarily linked to being a more potent stimulator of cytokine production.

The findings presented in this thesis indicate that it may well be possible to use serum from selected "high titre" blood donors to prepare intravenous IgG to treat patients suffering from pneumonia caused by a range of Gram-negative bacteria such as *Acinetobacter* spp, *Haemophilus influenzae*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. When the data was viewed it could be seen that some of the blood donors had a high level of response to the LPS of all the organisms. In general this showed that there were people who were able to mount an effective response to all the organisms and their serum would be the most appropriate to be used as it would provide the greatest protection from these organisms. Although such an approach is not new; it has been proposed for treating patients suffering from sepsis and other nosocomial infections (Braude *et al*, 1981; Ziegler *et al*, 1982) and for burns patients (Jones *et al*, 1980), it could also employ specific monoclonal antibodies (di Padova *et al*, 1993).

Future work could also investigate whether patients suffering from VAP show changes in anti-LPS antibodies similar to those seen in sepsis patients (Barclay *et al*,

1989), and those patients who have high levels of specific anti-LPS antibodies may be protected from developing VAP. If they do, active vaccination with a vaccine containing the relevant LPSs incorporated into liposomes would probably be most appropriate. This would be similar to the vaccine developed by Bennett-Guerrero and colleagues (Bennett-Guerrero *et al*, 2000a, Erridge *et al*, 2002a).

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